University of Alberta

GENE EXPRESSION IN BLASTOCYSTS AND THE ENDOMETRIUM DURING PORCINE PERI-IMPLANTATION DEVELOPMENT

by

Linghuo Jiang ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment

of the requirements for the degree of Doctor of Philosophy

in

Animal Science

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **GENE EXPRESSION IN BLASTOCYSTS AND THE ENDOMETRIUM DURING PORCINE PERI-IMPLANTATION DEVELOPMENT** submitted by Linghuo Jiang in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Animal Science.

Dr. Walter T. Dixon (Supervisor)

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Date October 4, 1999

Dedicated to

my wife, my daughter,

my parents, and my parents-in-law

for

their constant support during the course of this study.

ABSTRACT

Macrophage colony-stimulating factor (CSF-1) was found to be the major factor, in conditioned media of Jag-1 cells, responsible for stimulating porcine macrophage proliferation *in vitro*. A porcine CSF-1 cDNA was isolated and characterized from Jag-1 cells. The CSF-1 gene was found to be present as a single copy in the porcine genome. Expression of CSF-1 transcripts was up regulated in both blastocyst and endometrial tissues during the peri-implantation period in the pig, with higher levels in endometrial tissues than blastocyst tissues. Similarly, higher levels of c-fms (CSF-1 receptor) transcripts were detected in endometrial tissues than in blastocysts. Our data suggest that CSF-1 might exert both paracrine and autocrine effects on porcine pre-implantation embryonic development.

Expression of matrix metalloproteinase (MMP) transcripts and their regulators was examined in pregnant gilts during the peri-implantation period. In blastocysts, MMP-2, MMP-14 and TIMP-2 increased, while MMP-9 was undetectable. MMP-8, TIMP-1 and TIMP-3 transcripts also increased, whereas uPA exhibited a biphasic pattern of expression. In the endometrium, expression of MMP-1, MMP-2, MMP-8, MMP-9, MMP-11, MMP-14, uPA, TIMP-1, TIMP-2 and TIMP-3 were all observed, but not MMP-7 and MMP-13. ProMMP-2 and TIMP-2, but not proMMP-9, were detected in uterine flushings. A lack of MMP-9 expression in blastocysts might explain the non-invasive implantation seen in pigs. Co-expression of MMP-2, TIMP-2 and MMP-14 in blastocysts might contribute to extraembryonic mesoderm cell migration.

Expression of integrin subunit ($\alpha1$, $\alpha2$, $\alpha3$, $\alpha4$, $\alpha5$, αv, $\beta1$ and $\beta3$) transcripts was also determined during early gestation in pigs. In endometrial tissues, constant

expression of αv, β1 and β3 was detected, whereas α1, α2, α3 and α5 appeared to be developmentally regulated. Expression of α2, α3, αv, β1 and β3 exhibited similar patterns and was developmentally regulated in blastocysts, with high levels in 1-2 mm spherical blastocysts, low levels in 5-10 mm spherical blastocysts and variable levels in elongated blastocysts. In Day-28 placental tissue, moderate expression of α1, α3, αv and β3 and weak expression of α2 and β1 was observed. Jag-1 cells expressed high levels of α1, α2, α3, αv and β3 and low levels of α5 and β1. Our findings support an important role for integrins in the processes of early embryogenesis, embryo implantation and placentation in the pig. In summary, the present study has identified and characterized the expression of some important protein factors, in blastocysts and the endometrium, that are hypothesized to play critical roles in conceptus development during early gestation in the pig.

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TABLE OF CONTENTS

CHAPTER 1

LITERATURE REVIEW 1

INTRODUCTION 1

EARLY EMBRYONIC DEVELOPMENT IN THE PIG 2

A. Cleavage 2

B. Compaction and Blastocyst Formation 3

C. Formation of Germ Layers and Extraembryonic Membranes 5

MATERNAL RECOGNITION OF PREGNANCY 7

BLASTOCYST ELONGATION AND IMPLANTATION IN THE PIG 9

A. Blastocyst Expansion and Elongation 9

B. Blastocyst Attachment/Implantation 10

POSSIBLE MECHANISMS REGULATING BLASTOCYST ELONGATION AND IMPLANTATION IN THE PIG 12

A. Steroids and Their Receptors 13

B. Roles of Retinol and Retinoic Acid in Embryonic Morphorgenesis 15

C. Trophoblast-Uterine Epithelium Adhesion 16

D. Formation of Porcine Non-Invasive Placenta 20

E. Regulators of Implantation: Growth Factors and Cytokines 24

1. *Insulin-like growth factor family* 25

2. *Epidermal growth factor family* 26

3. *Transforming growth factor β family* 28

4. *Interferon family* 30

5. *Macrophage colony-stimulating factor* 31

6. *Leukemia inhibitory factor* 32

7. *Interleukin-1 family* 34

F. Other Factors Secreted in the Uterus during Peri-implantation Period....36

1. Uteroferrin....36

2. Kallikrein, kininogen and kinin system....36

POSSIBLE SIGNAL TRANSDUCTION PATHWAYS INVOLVED IN IMPLANTATION....37

A. Signal Transduction via Intracellular Receptors....37

B. Signal Transduction via Cell Surface Receptors....39

1. Ras-MAPK pathway....39

2. The JAK-STAT pathway....41

3. Integrin signal transduction....41

OBJECTIVES OF THE PRESENT STUDY....42

REFERENCES....46

CHAPTER 2

CSF-1/C-FMS IS AN IMPORTANT AUTOCRINE AND PARACRINE GROWTH FACTOR-RECEPTOR PATHWAY FOR REGULATING PROLIFERATION OF PORCINE TROPHOBLAST CELLS....80

INTRODUCTION....80

MATERIALS AND METHODS....82

A. Animals....82

B. Collection of Endometrial and Embryonic Tissues....82

C. Cell Culture....83

D. Cell Proliferation Bioassay....83

E. RNA Extraction....84

F. Primer Design and RT-PCR....84

G. Northern and Southern Blot Analysis....86

RESULTS....87

A. Cell Proliferation Bioassay....87

B. Cloning and Sequencing of Two overlapping Fragments of a Porcine CSF-1 cDNA sequence....87

C. The Coding Sequence and Deduced Amino Acid Sequence of the Porcine CSF-1 cDNA 88

D. Expression of CSF-1 in Jag-1 cells and Southern Blot Analysis of Porcine CSF-1 gene 89

E. Expression of CSF-1 transcripts in Endometrial and Embryonic Tissues during Pre-implantation Development 90

F. Expression of c-fms transcripts in Endometrial and Embryonic Tissues during Peri-implantation Development 91

DISCUSSION 92

REFERENCES 118

CHAPTER 3

EXPRESSION OF MATRIX METALLOPROTEINASES AND THEIR REGULATORS DURING PORCINE PERI-IMPLANTATION DEVELOPMENT 126

INTRODUCTION 126

MATERIALS AND METHODS 129

A. Animals 129

B. Collection of Cells, Blastocysts and Endometrial Tissues 130

C. RNA Extraction 130

D. Primers and RT-PCR 131

E. Substrate Gel Electrophoresis (Zymography and Reverse Zymography) 132

RESULTS 133

A. Assessment of Developmental Stages of Conceptuses 133

B. Expression of MMP-2 and MMP-9 134

C. Expression of MMP-14, uPA and TIMPs 134

D. Expression of Other MMP Transcripts 135

DISCUSSION 136

REFERENCES 155

CHAPTER 4

EXPRESSION OF INTEGRIN TRANSCCRIPTS IN BLASTOCYSTS DURING EARLY EMBRYOGENESIS, AND IN THE ENDOMETRIUM DURING EARLY GESTATION, IN THE PIG **166**

INTRODUCTION 166

MATERIALS AND METHODS 169

A. Animals 169

B. Collection of Blastocysts, Endometrial and Placental Tissues 169

C. Cell Culture and Neutrophil Collection 170

D. RNA Extraction 170

E. Primers and RT-PCR 171

RESULTS 172

A. Expression of Integrin Transcripts in Blastocysts 172

B. Expression of Integrin Transcripts in the Placental Tissue and Jag-1 cells 173

C. Expression of Integrin Transcripts in the Endometrium 173

DISCUSSION 174

REFERENCES 189

CHAPTER 5

GENERAL DISCUSSION AND CONCLUSIONS **196**

APPENDIX

EXPRESSION OF CYTOKINES IN BLASTOCYSTS DURING PERI-IMPLANTATION DEVELOPMENT IN THE PIG **221**

LIST OF TABLES

Table	Title	Page
1.1	Integrins in human uterine epithelium and trophoblast, and their ligands	18
1.2	Subunits of integrins and their ligands in porcine uterine epithelium and trophoblast	19
1.3	Matrix metalloproteinases and their substrates	21
1.4	Expression of IGF-I, IGF-II, IGF-IR, IGF-IIR and IGFBP-2 in the endometrium and the embryo of pigs	25
1.5	Expression of the EGF family in the oviduct, endometrium and embryo of pigs	29
1.6	Ligands activating the JAK and STAT pathway	40
2.1	Sequences of CSF-1 and c-fms primers designed in this experiment	97
2.2	The homologies of the coding sequences and deduced amino acid sequences of porcine, human and mouse CSF-1, and the positions of potential N-linked glycosylation (N-X-S/T) and glycosaminoglycan addition (acidic residues-S-G-X-G/A)	98
3.1	Determination of developmental stages of pregnant gilts and their conceptuses based on the onset of estrus and the peak of preovulatory LH surge, respectively	145
3.2	PCR primers for target genes and their sequences	146
3.3	PCR cycling conditions for amplification of target genes	147
4.1	Blastocyst and placental tissue samples used in this experiment	183
4.2	PCR primer sequences and predicted product sizes for integrin subunits	184
5.1	Cellular regulation of integrins, MMPs and TIMPs expression by growth factors/cytokines	201
6.1	Primers for cytokines and predicted sizes of PCR products	224

LIST OF FIGURES

Figure	Title	Page
1.1	Early embryo development in the pig	4
1.2	Endometrial secretion of $PGF_{2\alpha}$ in non-pregnant and pregnant pigs	8
1.3	Potential roles of growth factors and proteolytic enzymes during implantation in the pig	27
1.4	Action of steroid/thyroid hormone receptors	38
2.1	A). Stimulatory effect of Jag-1 cell-conditioned medium (CM) and inhibitory effect of YY106 hybridoma CM on the proliferation of porcine monocyte/ macrophages; B). Inhibitory effect of YY106 hybridoma CM on the proliferation of Jag-1 cells was in a dose-dependent fashion	99
2.2	The amplification of the 1,569-bp cDNA fragment from mRNA of Jag-1 cells	101
2.3	Northern blot hybridization analysis of RNA extracted from Jag-1, H69B and H69C cells	101
2.4	RT-PCR and restriction analysis of the 5' end region of porcine CSF-1 coding sequence	101
2.5	Nucleotide sequence and deduced amino acid sequence of porcine CSF-1	103
2.6	Comparison of amino acid sequences of porcine, human and mouse CSF-1	106
2.7	Southern blot hybridization analysis of Jag-1 cell DNA digested with restriction enzymes, *Bam* HI, *Eco* RI, *Hind* III and *Pst* I, respectively	108
2.8	Northern hybridization analysis of RNA extracted from endometrial tissues during pre-implantation development	110
2.9	Quantitative analysis of the 4-kb CSF-1 mRNA expression in endometrial tissues between Day 3 and Day 16.5	110
2.10	Comparison of CSF-1 mRNA expression levels between endometrial and embryonic tissues during pre-implantation development	112
2.11	A). Nucleotide sequence of the 303-bp fragment of porcine c-fms cDNA; B). Comparison of the deduced 112 amino acid sequences of porcine, human and mouse c-fms	114

2.12	A). RT-PCR amplification (45 cycles) of c-fms mRNA in porcine endometrium B-F). PCR amplification of CSF-1 and c-fm mRNA in porcine pre-implantation blastocysts	116
3.1	Diagram showing different points of time at which predicted onset of estrus and ovulation occur in relation to the peak of the LH surge	148
3.2	RT-PCR amplification of MMP-1, MMP-2, MMP-7, MMP-8, MMP-9, MMP-11, MMP-13, MMP-14, uPA, TIMP-1, TIMP-2, TIMP-3 and G3PDH transcripts in the endometrium during the peri-implantation period	149
3.3	RT-PCR amplification of MMP-1, MMP-2, MMP-7, MMP-8, MMP-9, MMP-11, MMP-13, MMP-14, uPA, TIMP-1, TIMP-2, TIMP-3, G3PDH & β-actin transcripts in blastocysts during the peri-implantation period	151
3.4	Gelatin zymographic and reverse zymographic analysis of MMPs and TIMPs in uterine flushings of gilts obtained on Days 3, 5.5, 7, 9, 10, 11 and 14 of pregnancy relative to onset of estrus, respectively	153
4.1	RT-PCR amplification of G3PDH and integrin subunits α_1, α_2, α_3, α_v, β_1 and β_3 in blastocysts, placental tissues and Jag-1 cells	185
4.2	RT-PCR amplification of G3PDH (A), α_1 (B), α_2 (C), α_3 (D), α_5 (E), α_v (F), β_1 (G) and β_3 (H) in the endometrium during early gestation	187
5.1	Schematic representation of the role of matrix matelloproteinase (MMP) system in implantation and early embryogenesis in the pig	207
5.2	Schematic representation of the hypothetical role of the integrin family in early embryogenesis and implantation in the pig	209
5.3	Characterization of the expression for IL-1, IL-6, IL-8, and IFN-γ transcripts in blastocysts during the peri-implantation period in the pig	211

LIST OF ABBREVIATIONS

CSF-1 or M-CSF	Macrophage colony-stimulating factor
c-fms	Receptor for macrophage colony-stimulating factor
CT	Cytotrophoblast
ECM	Extracellular matrix
EGF	Epidermal growth factor
FCS	Fetal calf serum
IFNγ	Interferon-γ
G3PDH	Glyceraldehyde-3-phosphate dehydrogenase
HBGF	Heparin binding EGF-like growth factor
HGF	Hepatocyte growth factor
IGF	Insulin-like growth factor
IL	Interleukin
INF	Interferon
Jag-1	Porcine trophoblast cell line
mRNA	Messenger ribonucleic acid
MGF	Macrophage growth factor
MMP	Matrix metalloproteinases
Mt-MMP	Membrane type-MMP
PDGF	Platelet-derived growth factor
PBS	Phosphate buffered saline
RP-FPLC	Reverse phase-fast protein liquid chromatography
RT-PCR	Reverse transcription-polymerase chain reaction
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
ST	Syncytiotrophoblast
TGF	Transforming growth factor
TIMP	Tissue inhibitor of metalloproteinase
TNF	Tumor necrosis factor
uPA	Urokinase-type plasminogen activator
VEGF	Vascular endothelial growth factor

CHAPTER 1 LITERATURE REVIEW

INTRODUCTION

During this century, increases in reproductive efficiency of livestock have mainly resulted from improved nutrition and management practices by increasing the frequency of complete reproductive cycles. Study of embryonic mortality in domestic species has become an increasing interest of research in recent years. Regardless of species, prolificacy is primarily regulated by number of eggs ovulated and fertilized, embryonic/fetal survival and neonatal survival (Bazer, 1994a). Reproductive performance in swine could be largely limited by ovulation rate and prenatal embryonic mortality, because fertilization rate in swine does not seem to limit reproductive efficiency (Van Der Lende et al., 1994; Ford and Youngs, 1993; Perry and Rowlands, 1962). However, inseminations performed too early or too late relative to ovulation reduced fertilization rates in sows (Kemp and Soede, 1997). Ovulation rate could be improved by increasing energy intake during late diestrus and proestrus, injection of exogenous gonadotropins, or by genetic selection (Wrathall, 1971).

Embryonic/fetal mortality in pigs may reduce potential litter size by 40 to 50% (Bazer, 1994a). Differences between parities may exist with prenatal mortality ranging from about 30% at parity 1 to 50% or more at higher parities (Foxcroft, 1997). It has generally been accepted that about 75% of the total prenatal loss occurs during the first 30 days of gestation (Ford and Youngs, 1993; Pope and First, 1985). However, even with high ovulation rates, lower embryonic mortality before Day 28 of pregnancy may occur in modern genotypes, resulting in loss of up to 50% of surviving embryos in the post-implantation period (Foxcroft, 1997). The underlying causes of embryonic mortality are

complex and could be derived from chromosomal aberrations in the embryo (Bishop, 1961; Day and Polge, 1968), genetic traits (Bishop, 1961; Perry and Rowlands, 1962), environmental factors (Wildt et al., 1975), nutrition (Foxcroft, 1997) and intrauterine infections (Scofield et al., 1974). However, a difference exists in embryonic mortality between Days 25 to 30 (30%) and Day 12 (5 to 10%), which suggests that considerable losses occur from Days 13 to 20 (Pope, 1994). This period of high embryonic mortality is coincident with blastocyst elongation and attachment in the pig (Perry and Rowlands, 1962), processes regulated *via* steroid and protein factors secreted from the endometrium and the embryo. Therefore, elucidating the mechanisms regulating blastocyst-endometrial interactions during blastocyst elongation and attachment will lend insight to aberrant processes of embryogenesis that may contribute to embryonic death in this important domestic animal.

In this literature review, embryonic development in early pregnancy in the pig will be briefly documented, followed by a discussion of the maternal recognition of pregnancy that is a critical step for prolonged maintenance of functional corpora lutea. Emphasis will be given to the possible mechanisms regulating blastocyst-endometrial interactions during blastocyst elongation and attachment in the pig. Relevant information from other mammalian species will be cited as needed.

EARLY EMBRYONIC DEVELOPMENT IN THE PIG

A. Cleavage

As in other mammalian species, the porcine egg is the largest cell, with the highest ratio of cytoplasmic to nuclear content in the female body, although because of the absence of yolk materials it is smaller than the eggs of other vertebrates such as fish,

amphibians, reptiles and birds. Fertilization activates a porcine egg to complete the second meiotic division and form a female pronucleus in the cytoplasm, which fuses with a male pronucleus derived from a penetrated sperm to produce a zygote (one-cell embryo). Immediately after the formation of the zygote, the fertilized egg undergoes several successive mitotic divisions, or cleavage, inside the zona pellucida without any increase in total mass (McLaren, 1972). The cells formed in the process of cleavage are known as blastomeres. In the pig, fertilization takes place in the ampulla of the oviduct, and the first cleavage normally occurs within 18 hr after ovulation, or around Day 2 of pregnancy (Day 0 is the onset of estrus) (Figure 1.1). The 2-cell stage of the embryo lasts for 6-8 hr, and the 4-cell stage takes 20-24 hr (Hunter, 1974; Flint, 1981). Porcine embryos move from the oviducts and into the uterine horns at about the 4-cell stage, i.e. Day 3 of pregnancy (Hunter, 1974; Bazer et al., 1982). Thereafter, further cleavage divisions follow until embryos reach the 16- or 32-cell stage in the uterus. Since the blastomeres are contained within the zona pellucida throughout the period of cleavage, the resultant group of blastomeres appears much like a solid ball of cells or a mulberry (Patten, 1948), and embryos at these stages are described as morulae.

B. Compaction and Blastocyst Formation

During late cleavage stages of embryos in the pig, blastomeres begin a process of compaction, by which loosely attached blastomeres become tightly packed together and joined by intercellular junctions. The first signs of compaction are seen as early as the 8-cell stage, or at Day 4 of pregnancy (Hunter, 1974). Compaction initiates the process of epithelial differentiation and, consequently, results in the formation of the ectoderm from the outer cell layer. The group of cells inside the ectoderm remains

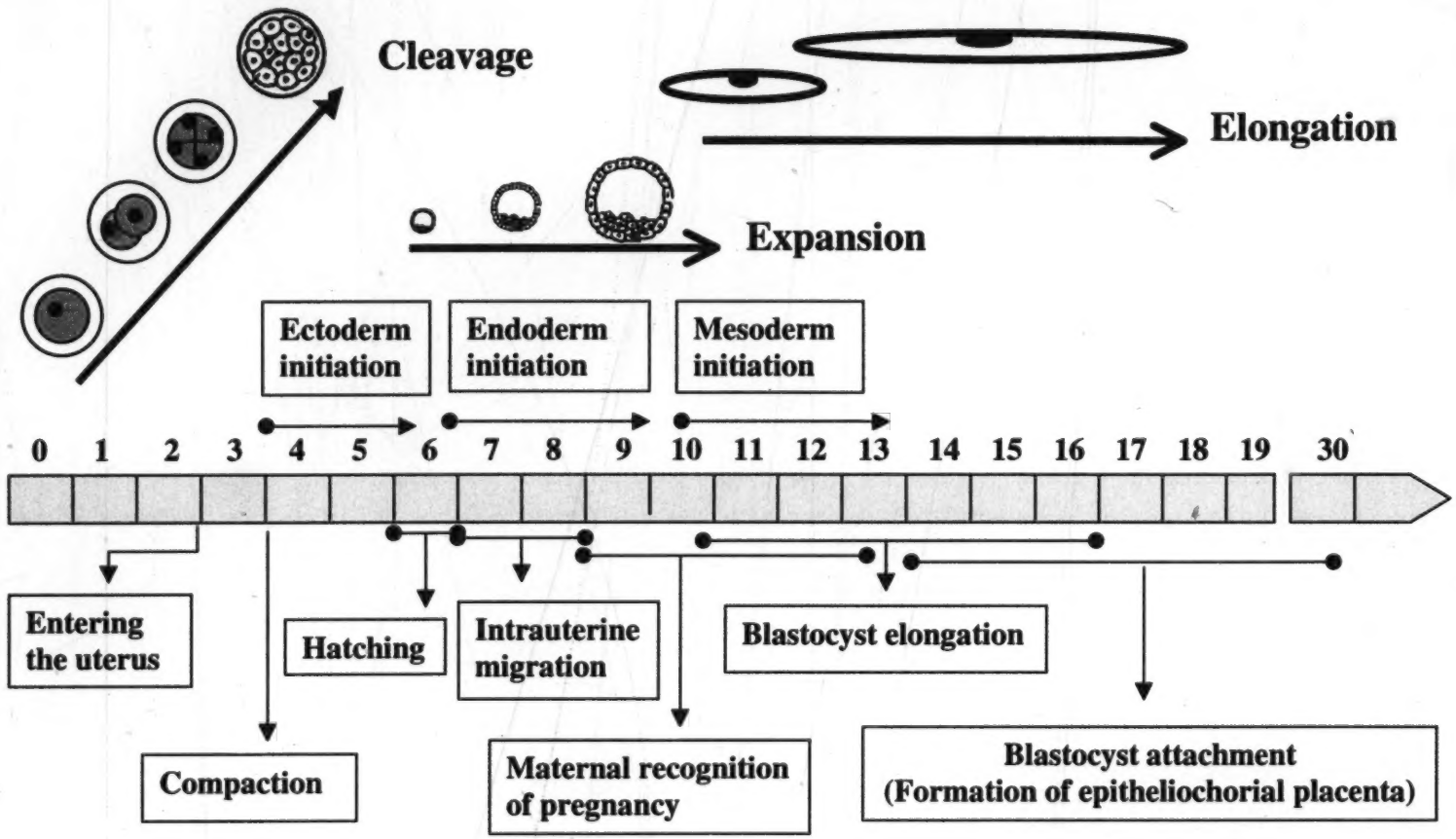

Figure 1.1 Early embryo development in the pig.

undifferentiated and is called the inner cell mass (ICM). Due to the inward fluid transport by the ectoderm cells, a fluid-filled cavity (blastocoele) appears within the morula, which marks the formation of the blastocyst by Day 5 (Papaioannou and Ebert, 1988). Once the blastocoele is established, it enlarges rapidly. Subsequently, blastocyst hatching from the zona pellucida occurs at Day 6-7 (Hunter, 1977), which may be caused by embryonic enzymes, mechanical mechanisms or uterine factors (Stone et al., 1986; Stroband and Van der Lende, 1990). The newly hatched blastocyst is about 0.2 mm in diameter (Geisert et al., 1982a).

C. Formation of Germ Layers and Extraembryonic Membranes

The first germ layer formed during embryonic development is the ectoderm, which consists of the embryonic **ectoderm** and the extraembryonic **trophectoderm** or **trophoblast**. As the hatched blastocyst expands, the ICM cells proliferate rapidly *in situ*. Some of the ICM cells form an outgrowth along the inner surface of the trophectoderm into the blastocoele (Patten, 1948; Geisert et al., 1982b), and subsequently form a complete layer of cells called the extraembryonic **endoderm** - the second germ layer (Patten, 1948; Barends et al., 1989). While the **endoderm** is being established, the embryonic ectodermal cells overlying the ICM degenerate; simultaneously, the ICM cells proliferate further and become aggregated into a disk-shaped and thickened area, which is sharply distinct from the adjoining trophectoderm. This area is called the **embryonic disc** (Patten, 1948; Geisert et al., 1982b).

Immediately after the endoderm has been established as a definite layer, the blastocyst undergoes a striking elongation from a spherical shape of about 10 mm in diameter to a filamentous shape of up to 100 cm in length (Anderson, 1978; Geisert et al.,

1982a; Stroband & Van der Lende, 1990). At about the time the blasotcyst begins elongation, a local differentiation occurs at one part of the margin of the embryonic disk, and the cells in this region undergo proliferation inward to the endoderm. Consequently, a longitudinal area appears as a thickened band over the embryonic disk area, which is known as the **primitive streak**. The proliferation and differentiation of the cells in the primitive streak region result in the formation of the third germ layer – the **mesoderm**, which subsequently spreads laterally to lie between the trophectoderm and the extraembryonic endoderm. The initiation of the mesoderm usually occurs on Day 9 of pregnancy in the pig (Patten, 1948) and immediately precedes the implantation process, which involves attachment between the trophoblast and the uterine epithelium.

Along with the development of the three germ layers, three extraembryonic membranes are formed in the pig (Perry, 1981). The trophectoderm fuses with the avascular outer layer of the extraembryonic mesoderm to form the **chorionic membrane**, which when vascularized supports the growth, nutrition, respiration and excretion of the embryo throughout the gestation. The **amniotic membrane** is also derived from the trophectoderm and the avascular outer layer of the extraembryonic mesoderm, and surrounds the embryo proper. The amniotic fluid can protect the embryo from desiccation and mechanical shock. An outgrowth of embryonic hindgut generates the allantoic membrane that consists of the extraembryonic endoderm and the vascular inner layer of the extraembryonic mesoderm. The allantoic fluid is connected with the urinary bladder of the fetus and is, therefore, the storage place of fetal waste products. As the allantois expands, it fuses with the chorionic membrane to form the **chorioallantoic placenta**.

MATERNAL RECOGNITION OF PREGNANCY

In luteal-dependent mammalian species like the pig, maintenance of the corpora lutea is essential during the whole gestation period. However, in all mammalian species, prolongation of luteal activity of pregnancy beyond the normal time of luteolysis is essential for the establishment of pregnancy and early embryonic development. Maternal recognition of pregnancy is the mechanism by which the developing conceptus signals its presence to the mother, resulting in the prolongation of functional activity of the corpus luteum and, therefore, the maintenance of pregnancy (Perry et al., 1973). Pregnancy recognition occurs before the embryonic tissue becomes intimately attached to the uterine epithelium, and is therefore distinct from implantation. The signal is prolactin in rodents (Cross et al., 1994) and chorionic gonadotropin in primates and horses (Niswender and Nett, 1988; Hearn et al., 1991), and those signals are luteotrophic. In some domestic mammals such as pigs, sheep and cows, the production of luteal progesterone is maintained not by luteotrophic factors, but rather by antiluteolytic factors that prevent the luteolytic effect of uterine prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), which triggers regression of the corpus luteum in non-pregnant animals. The signal is trophoblast-derived estrogen in the pig, and interferon-tau (IFN-τ) (previously named trophoblast protein-1) in sheep and cows (Bazer et al., 1997 and 1982; Geisert et al., 1994b).

There is little doubt that $PGF_{2\alpha}$, originating from the uterine endometrium, is the luteolytic factor that limits the lifespan of the corpus luteum during the estrous cycle (Bazer et al., 1986 and 1989). Upon receiving the pregnancy recognition signal from the developing embryo(s), an increase in $PGF_{2\alpha}$ synthesis by the endometrium is prevented in

sheep and cows; in contrast, endometrial production of $PGF_{2\alpha}$ in the pig is directed from the venous drainage (endocrine) to the uterine lumen (exocrine) (Figure 1.2; Bazer et al., 1982 and 1994b). However, the complete mechanism by which estrogen directs the secretion of $PGF_{2\alpha}$ into the uterine lumen during pregnancy is unknown. During the period of maternal recognition of pregnancy, porcine blastocysts undergo rapid expansion and initiation of elongation (Perry & Rowlands, 1962; Dhindsa & Dziuk, 1968; Anderson et al., 1978; Geisert et al., 1982b; Stroband and Van der Lende, 1990).

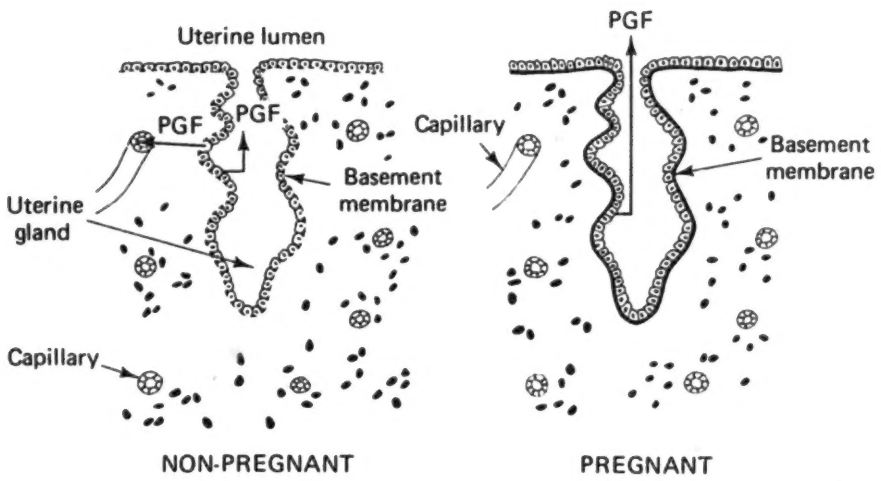

Figure 1.2 Endometrial secretion of $PGF_{2\alpha}$ in non-pregnant (left) and pregnant (right) pigs (Bazer et al., 1982). Upon receiving the pregnant signal from developing embryos, endometrial secretion of $PGF_{2\alpha}$ is directed from the venous drainage (endocrine) to the uterine lumen (exocrine).

BLASTOCYST ELONGATION AND IMPLANTATION IN THE PIG

A. Blastocyst Expansion and Elongation

In some mammalian species, blastocysts undergo expansion after they have hatched from the zona pellucida and before they proceed to implantation. The extent to which mammalian blastocysts expand is highly variable and species-dependent. The blastocysts of the human and laboratory rodents show little or no expansion before they implant either interstitially or eccentrically in the uterine endometrium. In contrast, the blastocysts of the rabbit, dog, cat and most livestock species increase their size by several orders of magnitude prior to central implantation (Biggers, 1972). In the extreme, blastocysts of the pig undergo pronounced expansion and subsequent elongation between Day 9 and Day 14 of gestation (Perry & Rowlands, 1962; Anderson, 1978). During this period, the porcine blastocyst transforms from a sphere of approximately 650 μm in diameter to a filamentous form that is up to 100 cm in length.

The capacity to synthesize estrogen is acquired when porcine blastocysts expand to the size of 5-6 mm in diameter, which is temporally associated with the formation of mesoderm (Fischer et al., 1985; Pusateri et al., 1990; Wilson and Ford, 1997). Blastocyst elongation appears to be programmed by endogenous development cues in the embryo, because blastocysts only elongate after they have reached the size of about 10 mm in diameter (Morgan et al., 1987a & 1987b). The 10-mm stage in conceptus development is coincident with the differentiation and expansion of extraembryonic mesoderm (Geisert et al., 1982b; Gupta et al., 1996), which is therefore believed to be the driving force for the cellular remodeling of blastocyst elongation (Geisert and Yelich, 1997). Upon reaching a diameter of about 10 mm on Day 11.5 of pregnancy, porcine

blastocysts undergo a rapid transition from spherical to tubular and filamentous forms (Geisert et al., 1982b), which precedes the attachment to the endometrium. During the initial elongation of pig blastocysts from Day 10 to 12, it appears that cellular remodeling rather than cellular hyperplasia is responsible for morphological changes (Geisert et al., 1982b). The actin cytoskeleton plays an important role in the modification of trophoblast cell shape in elongating porcine embryos (Mattson et al., 1990). Along with the elongation process, the biochemical and physiological activities of blastocysts are increased, which is evident by the onset of estrogen (Gadsby et al., 1980; Fischer et al., 1985) and protein synthesis (Godkin et al., 1982). The onset of embryonic estrogen synthesis is followed by a significant increase in intraluminal calcium content and a rapid release of secretory vesicles from uterine epithelial cells into the lumen (Geisert et al., 1982a; Stroband & Van der Lende, 1990).

B. Blastocyst Attachment/Implantation

Implantation of embryos in the wall of the uterus is the result of a series of complex interactions between the embryo and the uterus, which begins with the apposition of the blastocyst to the uterine epithelium and ends with the formation of a definitive placenta. The detailed process of implantation varies considerably in different species (Weitlauf, 1988), and can be divided into two steps, attachment and penetration. The attachment of embryos to the uterus involves a progressive interdigitation of microvilli between the trophoblast and the uterine epithelium, which occurs as two phases, apposition and adhesion. Adhesion between the two surfaces develops as the apposition phase progresses. Penetration (invasion) of the uterine wall by implanting embryos occurs in many mammals, such as the human, Chimpanzee, and laboratory

rodents (Weitlauf, 1988). However, porcine implanting embryos normally do not penetrate into the uterine wall and only remain attached to the uterine epithelium, as in other domestic species with an epitheliochorial type of placentation (Burghardt et al., 1997; Keys & King, 1990; Dantzer, 1985; Guillomot et al., 1981).

In the pig, intrauterine migration of embryos occurs between Day 7 and 12 of gestation (Dhindsa et al., 1967) (Fig. 1.1). The trophoblast and the uterine epithelium become closely apposed to each other as soon as blastocyst elongation is complete (Flood, 1991). The process of adhesion, beginning around Day 14, initiates in the region of the embryonic disc and progresses toward the trophoblastic tips (Dantzer, 1985; Keys and King, 1990). Based on morphological observations (Dantzer et al., 1981; Keys and King, 1990), the attachment between the blastocyst and the uterus is facilitated by the development of epithelial protrusions in the endometrium enclosed by trophoblastic caps. The formation of such structures is believed to serve to immobilize the elongated blastocysts. Adhesion between maternal and fetal epithelial cells is evident by the microvillous interdigitation between the uterine epithelium and the trophoblast by Day 16 (Dantzer, 1985; Keys and King, 1990). This process of attachment is accomplished over the entire surface of the embryo/conceptus by Day 26 of pregnancy (Amoroso, 1952). Discussion of the further development of the embryo proper is beyond the scope of this review.

POSSIBLE MECHANISMS REGULATING BLASTOCYST ELONGATION AND IMPLANTATION IN THE PIG

An essential step during mammalian pregnancy is the implantation of the blastocyst, which is initiated when the trophoblast attaches to the epithelium of the uterus. Before this point, the embryo develops during its migration from the fertilization site in the oviduct to the implantation site in the uterus. Thereafter, the developing embryo becomes dependent on the maternal environment for its continued development to term. Many factors have been implicated in the processes leading to the establishment of pregnancy and implantation in mammals, including hormonal and non-hormonal factors. Hormonal factors include progesterone, luteolytic factors and embryonic signals, which are responsible for maintaining the corpus luteum. Non-hormonal agents include adhesion molecules, vasoactive agents, tissue-remodeling enzymes [matrix metalloproteinases (MMPs)] and tissue inhibitors of MMP (TIMPs), growth factors and cytokines, which are involved in the pre-attachment period and the apposition, adhesion and invasion of the blastocyst.

Uterine secretion (uterotrophe) plays important roles during pregnancy in the pig. It is the major source of embryonic nutrition, since porcine trophoblasts are non-invasive and fail to make direct contact with the maternal blood supply. This type of nutritional exchange is relatively low in efficiency in comparison to the hemotrophe nutrition in species such as the mouse and human, but it is enhanced by the larger contact surface area formed between the trophoblast and the uterine epithelium in the pig. In addition, the formation of a non-invasive placenta in the pig is regulated by the components of uterine secretion together with the embryonic products.

A. Steroids and Their Receptors

One of the most important and earliest factors secreted by the pig embryo is estrogen, which is the signal for maternal recognition of pregnancy. Porcine blastocysts begin producing estrogen on Days 11-12 of gestation (Perry et al., 1976; Ford et al., 1982; Pusateri et al., 1990), a period characterized for the most diverse sizes of littermate conceptuses (Anderson, 1978; Pusateri et al., 1990). The expression of three enzymes involved in estrogen synthesis, $P450_{scc}$, $P450_{17\alpha}$ and $P450_{arom}$, has been detected in the trophectoderm (TE) cells of embryos with a minimum size of ~ 6 mm in diameter (Conley et al., 1992; Wilson and Ford, 1997). It appears that the rate-limiting enzymes in estrogen synthesis are $P450_{17\alpha}$ and $P450_{arom}$ in the pig conceptus, since expression of these two enzyme is closely linked with the estrogen content of the conceptus (Conley et al., 1992 & 1994; Ko et al., 1994; Green et al., 1995, Yelich et al., 1997a). At the time of blastocyst elongation, the receptor for estrogen is up regulated in the uterine epithelium (Geisert et al., 1993), whereas it is not detected in the early conceptus by RT-PCR (Yelich and Geisert, 1997). Therefore, it appears that the embryonic estrogen has an indirect effect on trophoblastic elongation. The brachyury gene, the marker for mesoderm differentiation (Herrman et al., 1990), is expressed coincidentally with the expression of $P450_{17\alpha}$ and $P450_{arom}$ (Yelich et al., 1997a), but its relationship with estrogen synthesis and blastocyst elongation is not yet clear.

During early pregnancy in the pig, morphologically advanced blastocysts appear to have a greater survival potential than their less developed littermates (Pope et al., 1982; Pope & First, 1985). The importance of synchrony between blastocyst development and uterine environment has been demonstrated through the application of

embryo transfer techniques in the pig (Geisert et al., 1991c). It is generally believed that estrogen derived from more advanced conceptuses changes the uterine milieu at the time of blastocyst elongation, which could be detrimental to less developed littermates (Dziuk, 1987; Pope et al., 1990; Pope, 1994). Indeed, embryonic estrogen has a modulatory role on the expression of some components of uterine histotrophe, such as retinol-binding protein (RBP) and uterine serpin (Trout et al., 1992) but not uteroferrin (Simmen et al., 1991). However, most of the major histotrophe components appear to be synthesized by the endometrium in response to progesterone (Roberts et al., 1993).

Differential production of embryonic estrogen has been proposed to be one of the factors that cause the Chinese Meishan breed to be more prolific than the American Yorkshire breed (Wilson and Ford, 1997). Chinese Meishan embryos are smaller and contain fewer trophectoderm cells than American Yorkshire embryos on Day 12 of gestation (Anderson et al., 1993; Rivera et al., 1996). Since estrogen synthesis occurs exclusively in the TE (Conley et al., 1994) and at a similar level per cell (Anderson et al., 1993), Meishan embryos produce much less estrogen than Yorkshire embryos on Days 11-12. Therefore, the uterine histotroph composition would be less dramatically changed in Meishans, allowing more embryos to survive (Wilson and Ford, 1997). This theory is also supported by evidence that there is less IGF-I in the uterine luminal fluid of the Meishan as compared to the Yorkshire and IGF-I might stimulate $P450_{arom}$ activity (Ko et al., 1994; Hofig et al., 1991a).

Expression of estrogen receptor (ER) mRNA in the endometrium of pregnant gilts was greatest on Day 10, followed by a decline on Day 15, and another increase on Day 18, but stayed low during the remainder of pregnancy (Geisert et al., 1993).

However, endometrial expression of progesterone receptor (PR) was highest on Days 0-5, decreased on Day 10, and reached a nadir on Day 12 that was maintained throughout the remainder of the estrous cycle (Geisert et al., 1994a). This dynamic change of PR and ER in the endometrium is consistent with the physiological role of embryonic estrogen in the maternal recognition of porcine pregnancy.

During blastocyst elongation, porcine embryos also produce PGF2α (Lewis and Waterman, 1983), although expression of the receptors for progesterone, estrogen, oxytocin and PGF2α have not been detected in embryonic tissues (Yelich et al., 1997b).

B. Role of Retinol and Retinoic Acid in Embryonic Morphogenesis

Retinol-binding protein (RBP) is present within the uterine lumen (Clawitter et al., 1990; Roberts and Bazer, 1988), and embryos (Trout et al., 1991; Harney et al., 1993) during rapid blastocyst elongation in the pig. Embryonic estrogen derived from more advanced embryos could trigger a massive increase in uterine expression and secretion of retinol-binding protein (Roberts et al., 1993; Trout et al., 1992). As a result, there would be an abrupt increase of retinol in the uterine lumen at a time when less developed embryos are least able to contend with excessive exposure to retinol, a potential teratogen (Lammer et al., 1985) and embryotoxic agent (Thompson et al., 1993).

RBP is believed to act as an acceptor and delivery vehicle of retinol from the plasma to its target tissues (Blomhoff et al., 1990), and also functions in the transport and regulation of cellular retinoids (Harney et al., 1994a & 1994b). One of the cytoplasmic metabolites, retinoic acid (RA) is known as an endogenous morphogenic substance (Ross, 1991). RA binds to a group of transcription factors called RA receptors (RARs),

which belong to the steroid/thyroid receptor family (De Luca, 1991) and consist of three subclasses, RARα, RARβ and RARγ (Mattei et al., 1988a and 1988b; Ishikawa et al., 1990). RARs specifically bind to RA response elements in the vicinity of target genes to activate gene expression (Linney, 1992). RA has been demonstrated to regulate the expression of tissue transglutaminase (Chiocca et al., 1989), extracellular matrix components (De Luca, 1991; Ross et al., 1994), proteases and their inhibitors (Adler et al., 1990; Tienari et al., 1991), adhesion molecules (Agura et al., 1992), and growth factors and their receptors (Jetten, 1980; Sporn et al., 1986). RA stimulates the expression of transforming growth factor β (TGFβ), which in turn acts as a chemoattractant to modify extracellular matrix components (Kimelman & Kirscher, 1987; Roberts and Sporn, 1988). Recently, Yelich et al. (1997) demonstrated that RBP, RARs and TGFβ3 are expressed temporally in elongating blastocysts during the peri-implantation period, suggesting their involvement in morphogenesis of porcine embryos. It is not surprising that RA has been implicated as one of the most promising candidates for embryonic tissue remodeling during blastocyst elongation (Roberts et al., 1993; Geisert and Yelich, 1997).

C. Trophoblast-Uterine Epithelium Adhesion

At implantation, the previously non-adhesive apical surface of the trophectoderm becomes adhesive. Increased knowledge of cell adhesion mechanisms, which occur during lymphocyte extravasation (Lasky, 1995), has led to extensive investigation of the molecular mechanism(s) underlying the adhesion process in the mouse and human. The present theory is that carbohydrate-lectin interactions might mediate initial blastocyst adhesion, which is then stabilized by binding of integrins to

their extracellular matrix (ECM) ligands (Aplin, 1997; Cross et al., 1994). The local loss of anti-adhesion molecules such as MUC-1 on the epithelial surface may contribute to the establishment of the receptive uterine state, and therefore facilitates attachment between the trophoblast and the uterine epithelium (Surveyor et al., 1995). Adhesion molecules implicated in the attachment process include integrins, ECM components, cadherins, lectins, heparin sulfate proteoglycans (Aplin, 1997; Lessey et al., 1996; Wegner and Carson, 1994; Cross et al., 1994; Damsky et al., 1993).

Integrins are a large family of cell surface glycoproteins involved in both cell-cell and cell-ECM interactions, and function as transmembrane heterodimers consisting of α and β subunits that are associated with cytoskeletal and cell signaling proteins (Gille and Swerlick, 1996; Hynes, 1992). At least 24 integrin heterodimers could be formed with the known 16 α subunits and 9 β subunits, and 9 of them have been identified in human uterine epithelium (Table 1.1; Aplin, 1997). Integrins are modulated during the reproductive cycle and early pregnancy in humans and rodents (Lessey et al., 1992; Tabibzadeh, 1992; Nishida et al., 1991), and peak expression of $\alpha v\beta 3$ and $\alpha 4\beta 1$ integrins in human uterine epithelium is coincident with the implantation window (Lessey et al., 1994). In the pig, some integrin subunits and ligands were detected in the uterine epithelium and trophoblast of estrous and early pregnant gilts (Table 1.2; Burghardt et al., 1997; Bowen et al., 1996 & 1997). Based on the spatial and temporal expression of integrin subunits and ligands at the implantation sites in the pig, integrins $\alpha 4\beta 1$, $\alpha 5\beta 1$ and $\alpha v\beta 1$, members of fibronectin receptors, have been implicated as the possible

Table 1.1 Integrins in human uterine epithelium and trophoblast, and their ligands

	Uterine epithelium	Trophoblast#	Ligands@
α1β1	+r*	e	LN,COL,PE
α2β1	+		COL
α3β1	+	e, v	FN,COL,LN
α4β1	+r		FN,VCAM
α5β1		e	FN
α6β1	?	?	LN
α9β1	+		TN
α6β4	+	v	LN
αvβ1	?	?	FN,VN
αvβ3	+r	e	FN,VN,OS,Vwf,FIB,BSP1,PE,PECAM-1
αvβ5	+		FN,VN,OS
αvβ6	+r		FN,TN

*r, regulated expression; #e and v, extravillous and villous expression, respectively; @BSP1, bone sialoprotein 1; COL, collagens; FIB, fibrinogen; FN, fibronectin; LN, laminins; OS, osteopontin; PE, perlecan (heparan sulfate proteoglycan); PECAM-1, platelet endothelial cell adhesion molecule; TN, tenascin C; VCAM, vascular cell adhesion molecule; VN, vitronectin; vWF, von Willebrand factor. '?' refers to integrins for which the constituent subunits are present but the specific association not demonstrated.

candidates for trophoblast-uterine epithelium attachment (Burghardt et al., 1997). In addition, expression of porcine MUC-1 in the uterine epithelium is down-regulated during the peri-implantation period as in humans and rodents (Burghardt et al., 1997; Aplin, 1997). Further studies are needed to determine which integrins are expressed specifically on the apical surface of porcine uterine epithelium.

It is now known that integrins serve as receptors for extracellular matrix (ECM) proteins and also transduce signals into the cell through focal adhesion kinase (FAK) (Yurochko, 1997; Juliano, 1996, Brown and Hogg, 1996). Integrin signaling events regulate cellular processes such as proliferation, apoptosis and motility (Cary and Guan, 1999). New evidence suggests that transmembrane-4 superfamily (TM4SF) proteins may facilitate integrin-signaling transduction by acting as linkers between extracellular integrin α subunit domains and intracellular signaling molecules (Hemler, 1998).

Table 1.2 Subunits of integrins and their ligands in porcine uterine epithelium and trophoblast#

	Subunits of integrins			Ligands
	H + C	H + M	L	
Uterine epithelium	αv; β3	α4; α5; β1	α1; α3	Vitronectin
Trophoblast	α1; α4; α5; αv; β1; β3			Vitronectin; Fibronectin
Sites of attachment	α4; α5; αv; β1; β3			Vitronectin; Fibronectin

#H+C, high and constitutive expression; H+M, high and modulated expression with maximal expression during maternal recognition of pregnancy (Days 10 to 15); L, low or no expression.

D. Formation of Porcine Non-invasive Placenta

Trophoblast cells are invasive in human and rodents, and produce two types of proteolytic enzymes, matrix metalloproteinases (MMPs) and a urokinase-type plasminogen activator (uPA), that degrade the ECM during endometrial invasion (Cross et al., 1994). At least 18 members have been characterized in the family of MMPs, which play important roles in wound healing, bone resorption, mammary involution, tumor invasion and matastasis, and pregnancy and parturition (Table 1.3; Parsons et al., 1997; Pei, 1999; Ryu et al., 1999; Pendas et al., 1997; Sato et al., 1997; Puente et al., 1996; Stolow et al., 1996). MMP-9 production and activation by both human and mouse trophoblasts peak during maximal invasive behavior *in vivo*, and are also required for trophoblast invasiveness *in vitro* (Harvey et al., 1995b; Shimonovitz et al., 1994; Behrendtsen et al., 1992; Librach et al., 1991). The uPA can activate the latent forms of matrix metalloproteinases (MMPs) through the PA-plasmin-MMPs cascade, but it does not appear to be essential for implantation (Carmeliet et al., 1994). MMP-9 has emerged as the main proteinase involved in implantation of human and mouse embryos (Alexander et al., 1996). Implantation in the pig is non-invasive and characterized by interdigitation between the trophoblast and the uterine epithelium, but porcine trophoblast becomes invasive when embryos are transferred to ectopic sites (Samuel, 1971 & 1972; Samuel and Perry, 1972). Furthermore, porcine embryos secrete uPA and MMP-2 during the peri-implantation period (Mullins et al., 1980; Fazleabas et al., 1983; Chamberlin and Menino, 1995), and very weak expression of uPA and MMP-9 transcripts is detected in the trophoblast (Menino et al., 1997). Trophoblast cells derived from porcine embryos of

Table 1.3 Matrix metalloproteinases and their substrates

MMP	Other name	Main substrate(s)
MMP-1	Collagenase-1	Fibrillar collagens
MMP-2	Gelatinase A (72 kDa)	Type IV and V collagens, fibronectin
MMP-3	Stromelysin 1	Laminin, fibronectin, non-fibrillar collagen
MMP-7	Matrilysin	Laminin, fibronectin, non-fibrillar collagen
MMP-8	Collagenase-2	Fibrillar collagens
MM-9	Gelatinase B (92 kDa)	Type IV and V collagens
MMP-10	Stromelysin 2	Laminin, fibronectin, non-fibrillar collagen
MMP-11	Stromelysin 3	Serpin (protease inhibitor)
MMP-12	Metalloelastase	Elastin
MMP-13	Collagenase-3	Fibrillar collagens
MMP-14	Mt1-MMP	ProMMP-2
MMP-15	Mt2-MMP	ProMMP-2
MMP-16	Mt3-MMP	ProMMP-2
MMP-17	Mt4-MMP	
MMP-18	Collagenase	Interstitial collagens
MMP-19		Gelatin
MMP-20	Enamelysin	Amelogenin
Mt5-MMP		MMP-2

Day 14 produce MMP-9 and –2 *in vitro* (Chai and Dixon, unpublished results). Therefore, the degradation of ECM components must be tightly controlled in order to support the formation of the noninvasive, epitheliochorial placenta in the pig, which could be accomplished by the endometrial secretion of a variety of protease inhibitors (Fig. 1.3).

Tissue inhibitors of MMPs (TIMPs), including TIMP-1, -2, -3 and –4, are the specific inhibitors for the MMPs (Fortunato et al., 1998; Leco et al., 1997). In the mouse, TIMP-3 transcripts were predominantly expressed in the decidua immediately adjacent to the embryo, and the expression of TIMP-1 and –2 was not detected at the sites of implantation (Harvey et al., 1995b; Reponen et al., 1995). Therefore, it appears that TIMP-3 is the principal endometrial inhibitor that regulates the extent of embryonic penetration in mice (Alexander et al., 1996; Leco et al., 1996). Porcine embryos and uteri also express TIMP-1, -2 and –3 during the period of blastocyst attachment. TIMP expression is abundant in porcine embryos, especially in the trophoblast, and is also localized in the stroma but not in the epithelium of the uterus (Menino et al, 1997). TGFβ is a potent inducer of TIMP-1 and –2 (Graham et al., 1994) and has been proposed as a negative regulator of trophoblast invasion (Cross et al., 1994). Both TGFβs and their receptors are produced in porcine embryos during the attachment period (see section on growth factors in this review). It is possible that embryonic production of TFGβ may participate in inducing TIMP expression in the uterus and/or strengthening the TIMP production in the trophoblast. Therefore, the formation of non-invasive placentae in the

pig could be mainly controlled from the embryos themselves by strong expression of TIMPs and TGFβ and weak expression of MMPs and uPA by the trophoblast.

The presence of inhibition of both uPA and plasmin in porcine uterine fluid could also nullify the potential extracellular proteolysis by uPA and plasmin (Mullins et al., 1980; Fazleabas et al., 1983). During blastocyst attachment in the pig, an inhibitor containing a Kunitz domain is secreted, which is specific for at least three serine proteases (plasmin, trypsin and chymotrypsin) (Duffy et al., 1997). Another group of plasma serine protease inhibitors, the inter-α-trypsin inhibitor (IαI) family, has also been detected in the endometrium during the elongation and attachment (Geisert et al., 1996; Diederich et al., 1997). The IαI family functions as complexes consisting of two types of subunits, heavy chain (H) and light chain. The light chain is also known as bikunin because it contains two Kunitz domains on its amino acid sequence. So far, three members of the IαI family has been identified, which include IαIH1- IαIH2-bikunin, IαIH2-bikunin and IαIH3-bikunin. The subunits in those inhibitors are connected to each other by inter-chain glycosaminoglycan bonds. In addition to binding to hyaluronic acid (HA), all IαI heavy chains contain a von Willebrand type-A domain that functions as a target for adhesion molecules such as integrins, collagen, proteoglycans and heparin. It is obvious that the IαI family functions not only as protease inhibitors but also as stabilizers for the ECM (Geisert and Yelich, 1997).

Two other protease inhibitors secreted by the porcine endometrium are antileukoprotinease (ALP) for a uterine elastase/cathepsin G protease (Simmen et al., 1992) and uteroferrin-associated proteins (UfAPs) that are related to the serpin family of

protease inhibitors (Murray et al., 1989; Malathy et al., 1990). The porcine endometrium also secretes many other enzymes including lysozyme, leucine-aminopeptidase, β-hexosaminidase, and cathepsins B1, D, E and L, which have anti-bacterial function and degrade proteins for embryo uptake (Geisert and Yelich, 1997). The cathepsins are lysozymal cysteine proteases that have been implicated as modulators of invasive implantation of the rat and cat (Elangovan and Moulton, 1980; Li et al., 1992). Cathepsin L, expressed in the porcine uterus, has a high affinity for collagen and elastin, which suggests that it may play a role in attachment between the trophoblast and the epithelium.

E. Regulators of Implantation: Growth Factors and Their Receptors

Growth factors are known to play important roles in the control of the processes of cellular proliferation, differentiation and morphogenesis during pre-implantation mammalian embryonic development, which has been extensively reviewed (Kaye PL, 1997; Kaye and Harvey, 1995; Schultz and Heyner, 1993). These growth factors are believed to function in an autocrine and/or paracrine fashion within the embryo itself or through interactions with the uterus. This review will not attempt to provide an exhaustive catalogue of these factors, but will focus on growth factor systems that are, or are likely to be, important during implantation in mammals, particularly in the pig. Recently, some of these growth factors have been demonstrated to be relevant to the process of implantation in mammals (Nie et al., 1997, Polan et al., 1995; Harvey et al., 1995a). Geisert and Yelich (1997) have reviewed the presence of growth factors and their receptors during the peri-implantation period in the pig, although little or no information exists at this point regarding their functions.

1. Insulin-like growth factor family

This family of growth factors has been extensively studied. Insulin-like growth factor-I (IGF-I) and IGF-II have a high degree of amino acid sequence homology with proinsulin, and are transported in extracellular fluids by binding to a group of IGF-binding proteins (IGFBPs). IGF-I receptor (IGF-IR) and IGF-IIR are structurally unrelated to each other, and IGF-IIR also carries a separate binding site for mannose-6 phosphate (M6P) (Kay, 1997).

Temporal expression patterns of IGF-I, IGF-II, IGFBP-2 and IGF-IR have been demonstrated in the endometrium of cyclic and pregnant pigs, and constitutive levels of IGF-II and IGF-IR are observed (Letcher et all, 1989; Simmen et al., 1992) (Table 1.4). In contrast, IGF-I and -II have also been detected in embryos, although little or no expression of IGFBP-2 is detected in embryos or placenta (Letcher et all, 1989; Simmen et al., 1992; Green et al., 1995). Expression of IGF-IR mRNA was demonstrated in peri-implantation embryos using a combination of RT-PCR and Southern hybridization

Table 1.4 Expression of IGF-I, IGF-II, IGF-IR, IGF-IIR and IGFBP-2 in the endometrium and the embryo of pigs.

	IGF-I	IGF-II	IGF-IR	IGF-IIR	IGFBP-2
Endometrium	+	+	+	-	+
Embryo	+	+	+	+	-

methods (Green et al., 1995), although it was not detected using less sensitive immunohistochemistry and autoradiography methods (Chastant et al., 1994). However, the expression of IGF-IIR was only observed in the trophoblast but not in the endometrium (Chastant et al., 1994). These results indicate that the IGF system modulates the interactions between the endometrium and embryos in a paracrine and/or autocrine fashion (Figure 1.3).

Endometrial expression of IGF-I mRNA and IGF-1 content in the porcine uterine lumen peak at Day 12 of pregnancy, which is concomitant with maximal estrogen production by embryos (Ko et al., 1994; Simmen et al., 1992; Letcher et al, 1989; Tavakkol et al., 1988). Moreover, $P450_{arom}$ levels in porcine embryos are highly correlated with the ratio of IGF-I to IGF-II in the uterine lumen (Ko et al., 1994). Furthermore, it has been demonstrated that induction of the functional activity of $P450_{arom}$ in porcine embryos by IGF-I is dependent on the development stage of these embryos *in vitro* (Hofig et al., 1991b). Therefore, endometrial production of IGF-1 might play a potential role in modulation of porcine embryonic steriodogenesis during the peri-implantation period.

2. Epidermal growth factor family

EGF is a small polypeptide of 53 amino acids, and stimulates the proliferation of epidermal and epithelial cells in whole animals and of a variety of cell types *in vitro*. Many polypeptides structurally and functionally related to the EGF have been described (Lim et al., 1997; Das et al., 1995), which include transforming growth factor-α (TFG-α), heparin binding-EGF (HB-EGF), amphiregulin (Ar), beta-cellulin, epiregulin, and

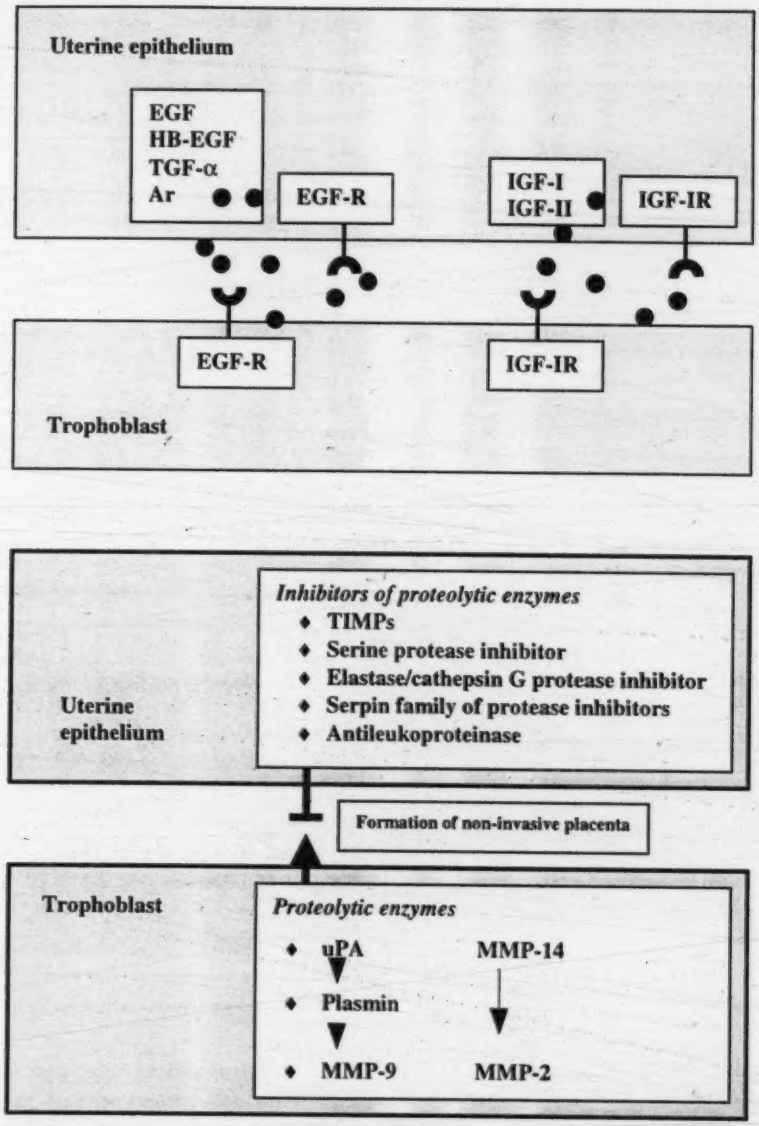

Figure 1.3 Upper panel, autocrine and paracrine actions of growth factors (EGF family and IGF-I family) at the interface between the trophoblast and the uterine epithelium during early pregnancy in the pig. Lower panel, production of proteolytic enzymes and their inhibitors during the attachment of the two surfaces, which might contribute to the formation of the non-invasive placenta in the pig.

heregulins. Four receptors for the EGF family are identified, which include erbB1 (EGF receptor), erbB2, erbB3, and erbB4. TGF-α and EGF have an equal affinity for the erbB1 (Lim et al., 1997). This family of growth factors have been implicated to be important for implantation in many species such as the human, mouse, rat, cow, sheep and cat (Yoo et al., 1997; Boomsma et al., 1997; Das et al., 1997; Johnson et al., 1996; Chia et al., 1995; Gharib-Hamrouche et al., 1995).

Expression of erbB1, EGF, TFG-α, and Ar at mRNA level, but not that of HB-EGF, has been detected in the oviduct and endometrium of cyclic and pregnant pigs (Kennedy et al., 1994) (Table 1.5). The erbB1 mRNA was detected at all stages of embryo development examined (Vaughan et al, 1992), but EGF expression started at Day 15 and continued to increase up to Day 22. Interestingly, TGF-α was expressed only at Day 8, 10 and 12, with a peak at Day 12, which is coincident with the maternal recognition of pregnancy in the pig. Recently, HB-EGF-like growth factors have been isolated from porcine uterine luminal flushings, which are expressed in endometrial tissues of pregnant and cycling pigs (Kim et al., 1995). In addition, high molecular mass forms (25 kDa) of epidermal growth factor have been detected in pig uterine secretions (Brigstock et al., 1996). These data indicate that this system of growth factor functions in an autocrine and paracrine manner during porcine blastocyst elongation and attachment (Fig. 1.3).

3. Transforming growth factor β

TGFβ is one member of a superfamily including activin and inhibin (Pfeilshifter, 1990). Three forms of TGFβ are identified, TGFβ-1, TGFβ-2 and TGFβ-3,

and two types of TGFβ receptors, TGFβ-IR and TGFβ-IIR, are found in most cells in different ratios. They mediate many key events in normal growth and development. In addition to its effect on cell proliferation and differentiation, TGFβ increases matrix synthesis and decreases matrix degradation by regulating the production of MMPs, TIMPs, tissue PA and PA inhibitor (Pfeilschifter, 1990). TGFβ is involved in mesoderm induction and body patterning in *Xenopus* and *Drosophila* embryos (Hoffman, 1991; Kimelman et al., 1992). Expression of TGFβ has also been demonstrated at implantation sites in the human, mouse and sheep (Graham et al., 1992; Dore, 1995; Stager et al., 1991), but little is known about their effects on mammalian embryo development.

Table 1.5 Expression of the EGF family in the oviduct, the endometrium and the embryo of pigs.

	Oviduct	Endometrium	Embryos							
			D7	D8	D10	D12	D15	D17	D18	D22
ErbB1	+	+	+	+	++	++	++	++	++	++
EGF	+	+	-	-	-	-	+	++	+++	++++
TFG-α	+	+	-	+	++	+	-	-	-	-
HB-EGF	-	-								
Ar	+	+								

TGFβ and its receptors have been shown to be differentially expressed during the peri-implantation period in the pig. Both TGFβ2 and TGFβ3 are expressed in the trophoblast from Days 10-14 of gestation when blastocysts undergo rapid elongation (Gupta et al., 1996). During this period, TGFβ-2 expression is dramatically decreased, whereas TGFβ-3 expression is constant. In porcine embryos, expression of TGFβ-3 is demonstrated to be developmentally regulated, and it increases gradually from the 2-mm spherical to 8-mm spherical stage and remains constant during the remainder of blastocyst elongation (Yelich et al., 1997b). In addition, TGFβ-IR is consistently expressed, although TGFβ-IIR expression is abruptly decreased in porcine embryos during the peri-implantation period (Gupta et al., 1996). TGFβs could be involved in initiation or progression of mesoderm migration in porcine conceptuses or in suppression of maternal immune reactions (Gupta et al., 1996).

4. Interferon family

Interferons (IFNs) are multifunctional cytokines, initially defined by their ability to induce a state of resistance against viral infection in target cells (Hauser, 1990). In mammals, they fall into two groups, type I and type II. Type I IFNs consists of a heterogeneous group of molecules sharing sequence homology, and they are encoded by a set of clustered, intronless genes and are expressed in a broad range of cell types. Until now, four subgroups of type I IFNs have been identified, which are IFNα, IFNβ, IFNω and IFNτ. IFNτ (previously called ovine trophoblastic protein) is the first member of the type I IFN family whose primary function is not antiviral and whose expression is physiologically controlled (Bazer et al., 1996). Type II IFN includes only IFNγ, and is

encoded by a single gene with introns. There are three types of receptors identified, IFNαB receptor, IFNα/β receptor (IFNα/β-R) and IFNγ receptor (IFNγ-R).

In the pig, all types of IFNs have been identified except of IFNτ (La Bonnardiere et al., 1994). IFN activity was first demonstrated to be expressed in embryos during Days 11 and 17 of pregnancy, and was also found in uterine flushings during the same period (Cross and Roberts, 1989). Upon further analysis, the major species responsible for the activity was shown to be IFNγ (Lefevre F. et al., 1990). Subsequently, a novel type I IFN gene was also isolated and characterized from a Day 15 pig embryo cDNA library (Lefevre and Boulay, 1993). The protein encoded by this novel gene is shorter and richer in Cys residues than other members of type I IFN family, and is distantly related to all of them. This gene is intronless in the pig genome, and, therefore, it represents the first member of a new subgroup of the type I family. It is unique that porcine trophoblast could be developmentally induced to produce both type I and type II IFNs. However, the precise roles of trophoblastic IFNs remains unknown, although a possible effect on implantation could be predicted by the fact that INFγ is known to up-regulate adhesion molecules in other cells (La Bonnardiere et al., 1994).

5. Macrophage colony-stimulatory factor

Macrophage colony-stimulatory factor (M-CSF or CSF-1) is a glycosylated protein with a molecular weight of 45 kDa (Das and Stanley, 1982) to 90 kDa (Wong et al., 1987) depending on the degree of glycosylation, and stimulates the survival, proliferation and differentiation of monocytes (Stanley et al., 1983). CSF-1 receptor (CSF-1R) is identical to the *c-fms* proto-oncogene product (Sherr et al., 1985), and is a

transmembrane protein with a molecular weight of 165 kDa. A role of CSF-1 in implantation was suggested by observations made in the CSF-1 deficient and osteopetrotic (csf^{op}/csf^{op}) mouse (Cohen et al., 1997; Pollard et al., 1991), which showed a lower implantation rate than wild type mice. These results indicate that the presence of CSF-1 is not essential for successful implantation in mice, which could be due to the functional redundancy of cytokines during the implantation period (Cohen et al., 1997).

In human, CSF-1 is expressed in the endometrium and blastocyst during the peri-implantation period (King et al., 1995; Kauma et al., 1991 and Arceci et al., 1989). The major source of CSF-1 was found to be decidual cells (Jokhi et al., 1995). CSF-1R has been predominantly localized to cytotrophoblastic cells (Jokhi et al., 1993), which suggests that CSF-1 is linked to trophoblast differentiation and proliferation (Hamilton, 1997). Furthermore, *in vitro* studies (Sapi et al., 1996) indicated that expression of CSF-1R could facilitate cellular invasion in mammary epithelial cells.

In the porcine endometrium, CSF-1 mRNA has been shown to increase gradually from Day 15 to term, with the highest level at term. Placental expression of CSF-1 also increased during the gestation stages examined with a peak between Days 20 and 30 (Tuo et al., 1995).

6. Leukemia inhibitory factor

LIF is a multi-functional cytokine and has been shown to regulate the proliferation and differentiation of many types of cells (Stewart, 1994; Shellard et al., 1996). In contrast, it promotes the proliferation but prevents the differentiation of embryonic stem cells *in vitro* (Smith et al., 1988). LIF is a member of a growing family

of cytokines, which includes IL-6, IL-11, Ciliary Neurotrophic factor (CNTF), Oncostatin M (OSM) and Cardiotrophin-1 (CT-1) (Shellard et al., 1996). Their transmembrane receptors are heterodimers and consist of LIF receptor chain and gp 130, which have low and high affinity with these ligands, respectively. The gp130 is responsible for signal transduction of all these family members (Kishimoto et al., 1992).

In mice, LIF expression in the endometrium is up-regulated at the time of blastocyst implantation (Bhatt et al., 1991). Furthermore, LIF null mice have been shown to ovulate normally, but their blastocysts fail to implant. However, their blastocysts are still viable, and could implant and develop to term if they are transferred to wild-type pseudopregnant recipients (Stewart et al., 1992; Shellard et al., 1996). In addition, *in vitro* studies have shown that LIF could down-regulate the production of uPA and MMP activities by mouse embryos from the peri-implantation period (Harvey et al., 1995a). Those results indicate that endometrial expression of LIF is required for the successful implantation. In the absence of LIF, neither embryo attachment nor decidualization of the uterus will occur (Stewart and Cullinan, 1997).

Peak LIF activities were observed in porcine uterine fluids on Day 12 of gestation as well as Days 7 to 13 of the estrous cycle, and IL-6 activity was detected throughout the pre-implantation period and the estrous cycle (Anegon et al., 1994). LIF mRNA was detected in the endometrium at Day 11 but not at Day 6, and it was not detected in the trophectoderm at Day 11. A porcine IL-6 cDNA was cloned, and IL-6 mRNA was first detected in porcine embryos at Days 13 and 21 of pregnancy (Mathialagan et al., 1992). Furthermore, higher levels of IL-6 mRNA were demonstrated in the endometrium than in embryos (Anegon et al., 1994). These results indicate that the

production of LIF and IL-6 is not dependent on the presence of embryos and is mainly derived from the endometrium. Further experiments are needed to clarify the temporal and spatial expression of LIF and IL-6 in the endometrium and embryos of pigs.

7. Interleukin-1 family

Interleukin-1 (IL-1) is a family composed of three structurally related polypeptides, IL-1α, IL-1β and IL-1 receptor antagonist (IL-1ra) (Simon et al., 1995 & 1997). IL-1α and IL-1β recognize the same receptor on target cells and produce similar effects (Dower et al., 1986), although, surprisingly, they share only 26% amino acid homology (Veerapandian, 1992). There are two types of receptors for IL-1, type I (IL-1RI) and type II (IL-1RII), and they function independently at the level of ligand binding because they do not form a heterodimer (Slack et al., 1993).

In both mouse and human endometrium, IL-1α and IL-1β have been localized at the mRNA and protein levels to endometrial macrophages and endothelial cells (Takacs et al., 1988; Simon, et al., 1993). Similar dynamics of IL-1 production were observed in the endometria of pregnant and pseudopregnant mice (Choudhuri and Wood, 1993; De et al., 1993), which suggests that the endometrial IL-1 is hormonally regulated. The IL-1 system has also been shown to present in human and mouse embryos (Kruessel et al., 1997; De los Santos et al., 1996). However, the embryo releases the IL-1 only when it is co-cultured with human endometrial cell-conditioned media, which indicates an obligate role of the endometrium in the regulation of the embryonic IL-1 system (Simon et al., 1997).

Earlier in the development at the human implantation site, IL-1RI was located in the syncytiotrophoblast (ST) and maternal decidua, whereas IL-1β was present in the ST, villous and columnar cytotrophoblast and maternal decidua. IL-1ra was also observed in the intervillous space and maternal decidua (Hu et al., 1992; Simon et al., 1994a). These results suggest that the IL-1 system acts in an autocrine/paracrine manner in human implantation. In addition, *in vitro* studies demonstrated that IL-1 had a stimulatory effect on hCG release from human first-trimester trophoblast cells (Yagel et al., 1989). Therefore, the IL-1 system may exert autocrine/paracrine regulatory effects on hCG secretion by the stimulation of IL-1RI located on ST.

From the studies above, it seems that under the proper progesterone-primed environment, endometrial IL-1RI is synthesized and in turn triggers the production of IL-1 in the trophoblast of the implanting embryo. The interaction between embryonic IL-1 and endometrial IL-1R somehow initiates a series of events, which allows the embryo to attach and invade through the uterine epithelium and basement membrane (Polan et al., 1995). Such implantation events in mice are blocked by the introduction of IL-1ra into the uterine cavity (Simon et al., 1994b), which supports the hypothesis that appropriate interactions between the IL-1 and its receptor at the endometrium and embryo interface are a requisite for successful implantation. However, conflicting results were obtained from studies on mice lacking functional IL-1RI, which did not show any profound alterations in their reproduction (Abbondanzo et al., 1996). Furthermore, the effect of IL-1ra on implantation was not repeated in either wild type or IL-1RI deficient mice.

Recently, IL-1 has been shown to augment the production of LIF in mouse decidual cells (Sawai et al., 1997), IL-6 in human endometrial epithelial cells (Laird et

al., 1994) and CSF-1 in placenta cells (Kauma, 1993). All of these growth factors are also implicated to play a role during implantation, which indicates that a network of growth factors and cytokines might exist in the endometrium to influence embryo implantation.

In the pig, expression of IL-1β has been examined at the mRNA and protein level in embryos recovered from Day 11 to Day 12 of pregnancy (Tuo et al., 1996). The production of IL-1β was observed in porcine peri-implantation embryos of Days 11 to 13 of pregnancy, but not in conceptuses recovered after Day 15 of pregnancy. Further characterization of the IL-1 family in conceptus and endometrium is needed to understand the potential role of IL-1 for the establishment of pregnancy and/or implantation in the pig.

F. Other Factors Secreted in the Uterus during the Peri-Implantation Period

1. Uteroferrin

Uteroferrin is one of the earliest uterine proteins identified within the porcine uterus (Roberts et al., 1986; Roberts and Bazer, 1988). In addition to playing a role in iron transport to the fetus throughout pregnancy, uteroferrin may also serve as a hemotopoietic stem cell growth factor during early embryo development (Bazer et al., 1991; Geisert and Yelich, 1997).

2. Kallikrein, kininogen and kinin system

The activity of kallikrein, one type of kininogenase, is found to increase during the elongation period, which indicates that the kallikrein-kininogen-kinin system might play a role in the trophoblast attachment to the uterine epithelium (Geisert and Yelich,

1997). Kallikreins are serine proteases, and their substrates are kininogens (Bhoola et al., 1992). Kinins are the cleavage products of kininogens, and include bradykinin, kallidin (Lys-bradykinin) and Met-Lys-bradykinin. Kinins are vasoactive peptides that influence many biological processes. They are hypotensive, increase vascular permeability, contract smooth muscle of the intestine and uterus, and increase sperm motility.

In the pig, embryonic estrogen stimulates increases in uterine blood flow, vasodilatation of the capillaries surrounding elongated blastocysts, contraction of the myometrium, and endometrial release of calcium, protein and histamine (Geisert et al., 1990). These processes could be mediated by the kallikrein-kininogen-kinin system in the pig uterus (Geisert and Yelich, 1997).

POSSIBLE SIGNAL TRANSDUCTION PATHWAYS INVOLVED IN MAMMALIAN IMPLANTATION

A. Signal Transduction via Intracellular Receptors

Steroids (progesterone, androgen, and estrogen) can directly diffuse across the plasma membranes of target cells, and their biological effects are mediated by intracellular ligand-activated transcription factors, which also include receptors for non-steroids such as thyroid hormone, retinoids and vitamin D, as well as orphan receptors whose ligands and/or functions are unknown (Weigel, 1996). In the absence of ligands, steroid receptors are associated with heat shock proteins (HSPs) of 90 kDa and 70 kDa (Smith, 1993). Engagement of the receptors to steroids leads to the dissociation of the HSPs and receptor dimerization (Figure 1.4). As a result, the dimerized receptor binds to

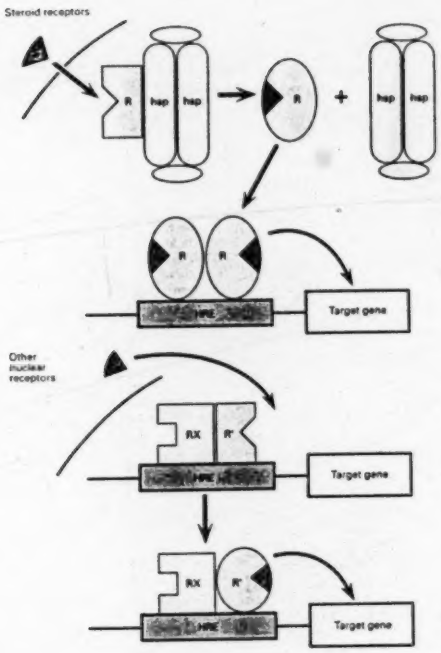

Figure 1.4 Action of steroid/thyroid hormone receptors (Weigel, 1996). Upper panel depicts activation of steroid receptors. In the absence of steroids, each receptor (R) monomer is associated with a HSP complex. Binding of steroids (S) causes a conformational change and results in dissociation of the HSP complex and receptor dimerization. Consequently, dimerized receptors bind to a hormone response element (HRE) of the target gene and activate the transcription of the gene. Lower panel describes the action of non-steroid receptors (R'). In the absence of ligands, R' typically forms a heterodimer with retinoid X receptor (RXR), which binds to HRE of the target gene and repress the transcription of the gene. Binding of hormone (H) activates the receptor and causes the activation of gene transcription.

a specific steroid response element of target genes, and subsequently interacts with basal transcription factors, other DNA binding proteins or co-activators, resulting in transcription of these genes (Weigel, 1996; Ing et al., 1992; Schule et al., 1988a and 1988b). In contrast, the receptors in this family for non-steroids typically form heterodimers with retinoid X receptors (RXRs) in the nucleus, where they are tightly bound to specific response elements of target genes in the absence of ligands and repress the transcription of these genes (Mangelsdorf et al., 1990; Leid et al., 1992; Yu et al., 1991) (Fig. 1.4). Ligand binding may cause dissociation of repressor molecules and allow activation of transcription of target genes (Weigel, 1996; Horlein et al., 1995; Kurokawa et al., 1995).

B. Signal Transduction via Cell Surface Receptors

Engagement of cell surface receptors for growth factors and cytokines initiates a cascade of signal events, which causes complex cytoplasmic and nuclear responses. There are many signal transduction pathways mediated by different types of receptors. Here are a few major pathways possibly involved in mammalian implantation.

1. RAS-MAPK pathway

Many receptors for cytokines and growth factors contain an intracellular protein tyrosine kinase (PTK) or associate with non-receptor PTKs containing SH2 and SH3 domains. Ligand binding induces the receptor to dimerize, which activates the kinase activity of either the receptor or its associated PTK (Heldin, 1995 and 1996). When activated, receptor tyrosine kinases usually cross-phosphorylate themselves on multiple tryosine residues, which then serve as docking sites for downstream signaling proteins.

Table 1. 6 Ligands activating the JAK and STAT pathway

Family	Members
IFN	IFNα, IFNγ, IL-10
Receptor tyrosine kinases	EGF, CSF-1, SCF, PDGF
gp130	LIF, IL-6, IL-11, IL-12, CNTF, G-CSF, OnM
gp140	IL-3, IL-5, GM-CSF
Seven transmembrane receptor	Angiotensin II
IL-2	IL-2, -4, -7, -9, -13, -15
Growth hormone (GH)	GH, prolactin, erythropoietin, thrombopoietin

Ras protein is active when bound to GTP and inactive when bound to GDP. The activation state of Ras protein is regulated by GTPase activating protein (GAP) (negative regulator) and guanine nucleotide exchange factor (Sos) (positive regulator) (Lowy and Willumsen, 1993). Autophosphorylated tyrosine kinase receptors could recruit and activate the complex of adaptor protein Grb2 and Sos directly (or through another adaptor Shc). This causes translocation of Sos to the plasma membrane where Ras is located, and hence increases the rate of nucleotide exchange on Ras leading to its activation (Downward, 1996). Subsequently, activated Ras triggers an activation cascade of serine/threonine kinases including Raf-1, MEK (MAPKK) and MAPK. Activated MAPK is translocated into the nucleus, where it phosphorylates certain transcription factors. This pathway is important for stimulation of cell growth.

2. The JAK-STAT pathway

Molecular characterization of IFN-induced gene activation led to the discovery of the JAK-STAT signal transduction pathway, which is now known to be shared by other cytokines, growth factors and hormones (Williams and Haque, 1997; Shauai, 1994; Ihle et al., 1994). Binding of these ligands to their transmembrane receptors result in activation of members of the Janus family tyrosine kinase (JAK). The activated receptor-kinase complexes recruit members of the signal transducers and activators of transcription (STAT). As a consequence, the phosphorylated STAT proteins dimerize, translocate into the nucleus, bind response elements of target genes and activate the transcription of these genes. The specificity of biological effects is determined by the specific activation of members of the JAK and STAT families by the ligand. The ligands identified to activate the JAK and STAT pathway are listed in Table 1.6 (Heim, 1996).

3. Integrin signal pathway

Integrin-mediated signaling is also a complex process that shares some characteristics with those of growth factors and cytokines, including receptor cross-linking, up-regulation of tyrosine phosphorylation and activation of MAPK (Yurochko, 1997). However, integrin engagement causes the phosphorylation of a non-receptor tyrosine kinase named focal adhesion kinase (FAK), which may activate other SH2-containing kinases including Src, Fyn and phosphatidyinositol 3-kinase. Adhesion-induced activation of MAPK and the association of Grb-2 with FAK suggest that integrins might activate the Ras-MAPK signal pathway (Chen et al., 1994; Schlaepfer et al., 1994). However, integrin signaling to MAPK seems to be independent of Ras (Juliano, 1996).

In addition, the cytoskeleton could play a role in most aspects of integrin signaling since it is associated with integrins and FAK at focal contacts, which may be a mechanism for localizing some of the important factors in the signal transduction cascade (Yurochko, 1997; Juliano, 1996). Furthermore, other transmembrane proteins such as TM4 superfamily proteins (CD9, CD63 and CD81) are also involved in integrin signaling (Berditchevski et al., 1997; Shaw et al., 1995). It is clear that there is a lot about integrin signaling that remains to be worked out.

OBJECTIVES OF THE PRESENT STUDY

In the pig, embryonic mortality within the first 30 days of gestation could reduce potential litter size by 30 to 40%, and considerable losses occur from Days 13 to 20, coincident with blastocyst elongation and implantation (attachment). Early embryogenesis and implantation are two critical processes that occur during the peri-implantation period in the pig. To date, little is known about the mechanisms regulating early embryogenesis and implantation in the pig. Understanding these mechanisms might allow insights to aberrant processes of embryogenesis that may contribute to embryonic death in this important domestic animal. The overall objective of this study was to characterize the gene expression of important protein factors in blastocysts and the endometrium during the peri-implantation period.

OBJECTIVE 1 To determine the identity of Jag-1 macrophage growth factor (MGF) and to characterize the expression of this growth factor (s) in blastocysts and the endometrium during pre-implantation development in the pig.

Rationale Growth factors/cytokines are a group of protein factors, which are directly involved in the communication between the embryo and the endometrium during early mammalian embryo development (Simon et al., 1998; Schultz and Heyner, 1993). They have also been demonstrated to be important cellular regulators for MMP, TIMP and integrin production and activity *in vitro* (Sehgal and Thompson, 1999; Bischof et al., 1998; Parsons and Parsons, 1997; Harvey et al., 1995b). A trophoblast cell line, Jag-1, was previously isolated from Day-14 porcine embryos and was shown to produce growth factor (s) that could stimulate the proliferation of porcine macrophages *in vitro* (Ramsoondar et al., 1993). Upon reaching a size of about 10mm in diameter on Day 10 of pregnancy, porcine blastocysts undergo extensive elongation (Geisert and Yelich, 1997). This elongation process requires both the rapid proliferation of the trophoblast and cellular reorganization (Geisert et al., 1982b). Therefore, characterization of the macrophage growth factor (s) might help us understand the blastocyst elongation process. A previous study by Ramsoondar (1994) demonstrated that a protein species with a size of approximately 40 kDa was associated with the bioactivity and a strong hybridization signal to Jag-1 cellular RNA was obtained by Northern blot analysis with a probe for human CSF-1 (36-45 kDa). Therefore, **the hypothesis for the first experiment** of the present study was that **CSF-1 was the candidate for Jag-1 macrophage growth factor (MGF)**. This subject is addressed in Chapter 2.

Experimental approach 1) Examine the inhibitory effect of an anti-human CSF-1 monoclonal antibody on the proliferation of porcine macrophages by the Jag-1 MGF. 2) Clone the cDNA sequence of the MGF from Jag-1 cellular RNA. 3) Determine the copy number of the MGF gene in the porcine genome. 4) Examine the expression of the MGF in blastocysts and the endometrium during pre-implantation development in the pig using a RT-PCR approach.

OBJECTIVE 2 Mechanism regulating embryo implantation in the pig.

Rationale Embryo implantation during pregnancy in pigs is a non-invasive process that results in simple attachment between the trophoblast and the uterine epithelium. This type of placentation is termed epitheliochorial to distinguish it from other more invasive types of placentation, such as the hemochorial placentation seen in rodents and humans. However, when porcine embryos were artificially transplanted to ectopic sites, the trophoblast cells appeared to be highly invasive (Samuel and Perry, 1972a&b). This suggests that either the environmental cues present at the ectopic site caused these cells to become invasive or, alternatively, that the intrinsically invasive nature of these cells must be regulated *in vivo* during normal conceptus development. **The hypothesis for the second experiment** of the present study was that **the balance between ECM degradation and deposition in the embryo and at the interface between the trophoblast and the uterine epithelium must be tightly controlled**, during the peri-implantation period, such that implantation and early embryogenesis can proceed appropriately. This subject is addressed in Chapter 3.

Experimental approach 1) Characterize the temporal expression of MMP, TIMP and uPA transcripts in blastocysts and the endometrium during the peri-implantation period using a RT-PCR approach. 2). Examine the presence of proMMPs and TIMPs in uterine fluids prior to implantation using zymography and reverse zymography techniques.

OBJECTIVE 3 To examine the expression of integrin transcripts in blastocysts and in the endometrium during the peri-implantation in the pig.

Rationale Early embryogenesis and implantation involve extensive cell-cell and cell-extracellular matrix interactions, which are mediated through adhesion molecules that include Muc-1, heparin sulfate proteoglycans, lectins, cadherins, ECM components, and integrins (Aplin, 1997). Integrins are critical for signal transduction from the ECM into the cell and play an important role during early embryogenesis, implantation and placentation in the mouse (Coutifaris et al., 1998; Damsky et al., 1997). The presence of some integrin subunits was demonstrated in previous studies by Bowen et al. (1996 & 1997). **The hypothesis for the third experiment** of the present study was that **the expression of integrins must be developmentally regulated in blastocysts and the endometrium during the peri-implantation period** in order to facilitate cell migration during extraembryonic endodermal and mesodermal formation, and the attachment between the trophoblast and the uterine epithelium during implantation in the pig. This subject is addressed in Chapter 4.

Experimental approach Examine the expression of integrin transcripts in blastocysts and the endometrium during the peri-implantation in the pig using a RT-PCR approach.

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CHAPTER 2

CSF-1/C-FMS IS AN IMPORTANT AUTOCRINE AND PARACRINE GROWTH FACTOR-RECEPTOR PATHWAY FOR REGULATING PROLIFERATION OF PORCINE TROPHOBLAST CELLS

INTRODUCTION

Macrophage colony-stimulating factor (CSF-1) is a glycoprotein which was initially identified as a hematopoietic growth factor involved in the proliferation and differentiation of monocytic cells in both the human and the mouse (reviewed in Stanley et al., 1983 and Stanley, 1990). CSF-1 has since been shown to be involved in a host of diverse biological activities, mediated through its cell surface tyrosine kinase receptor (CSF-1R) (the product of the *c-fms* protooncogene) (Sherr et al., 1985). These include the regulation of early embryonic and placental development (Pollard, 1987 and 1990; Pampfer et al., 1991), uterine growth and function prior to and during gestation (Pampfer et al., 1991; Bartocci et al., 1986; Arceci et al., 1989; Pollard, 1991; Daiter et al., 1992), as well as a role in the pathogenesis of human carcinomas (Filderman et al., 1992). More recently, studies on CSF-1 nullizygous mice ($csfm^{op}/csfm^{op}$) have revealed other important functions of CSF-1 in gametogenesis, gonadal steroidogenesis and mammary gland development during pregnancy (Sapi and Kacinski, 1999; Cohen et al., 1996 & 1997; Pollard 1997). In addition, the expression of CSF-1 and its receptor has been positively correlated with the invasiveness of tumors of the human breast, ovary, and endometrium (Sapi et al., 1996; Kacinski, 1997).

In both the human and the mouse, the CSF-1 gene is present as a single-copy in the genome (Kawasaki et al., 1985; Ladner et al., 1987; Rajavashisth et al., 1987). The human *csf-1* consists of 10 exons and 9 introns, which are present on a genomic DNA fragment of about 21 kb situated on the long arm of chromosome 5 (Kawasaki and

Ladner, 1990; Pettenati et al., 1987). The human CSF-1 gene is expressed in many types of tissues and cells and in many cases transcript size heterogeneity has been observed. Of at least seven species of mRNA detected in human cells, five cDNAs corresponding to the 1.6-, 2.2-, 2.5-, 3.0-, and 4.0-kb mRNA species have been cloned (Kawasaki et al., 1985; Wong et al., 1987; Ladner et al., 1987; Cerreti et al., 1988; Pampfer et al., 1991). Three isoforms of a CSF-1 protein precursor could be deduced from the five cDNA sequences characterized, which are CSF-1^{554} [554 aa in length], CSF-1^{438}, and CSF-1^{256} (Fixe and Praloran, 1997; Deng et al., 1996). The first two precursors are ultimately secreted as soluble growth factors, while the shortest precursor is membrane-bound and externalized at the cell surface, where it is slowly released by extracellular proteolysis (Rettenmier et al., 1987; Pampfer et al., 1991). In the mouse, cDNAs of two major mRNA species (2.3 and 4.0 kb) of mouse CSF-1 have been cloned (Ladner et al., 1988). The coding sequence of rat CSF-1 cDNA has been shown to be identical to that of mouse CSF-1 (Borycki et al., 1993).

The human c-fms proto-oncogene is about 75 kb in length, consisting of 22 exons and 21 introns, which is also located on the long arm of chromosome 5 (Sherr, 1990; Groffen et al., 1983). Exon 1 is transcribed in placental cells but not in macrophages due to the differential transcription of the c-fms gene by two different tissue-specific promoters, and multiple initiation sites for transcription appear to be utilized in placental cells (Visvader and Verma, 1989). Expression of CSF-1R mRNA, but not CSF-1 mRNA, was detected at low levels in mouse embryos throughout pre-implantation development (Arceci et al., 1992).

Using a human CSF-1 cDNA and an anti-human CSF-1 monoclonal antibody, the expression of CSF-1 at both the mRNA and protein level were demonstrated in porcine uterine, conceptus, allantochorion and fetal tissues during post-implantation stages of pregnancy (Tuo et al., 1995). However, to date, there has been no published information on the sequence of porcine CSF-1 cDNA. In this study, we provide evidence that CSF-1/c-fms is an important autocrine/paracrine growth factor-receptor pathway for regulating proliferation of porcine trophoblast cells. In addition, we describe for the first time the cDNA sequence encoding porcine CSF-1, demonstrate that the CSF-1 gene is present as a single copy in the porcine genome, and examine the mRNA expression of CSF-1 and c-fms in endometrial and embryonic tissues during pre-implantation development.

MATERIALS AND METHODS

A. Animals

Sexually mature gilts (PIC Camborough) of similar age and weight and having two recorded estrous cycles were heat-checked three times daily (08:00, 16:00 and 24:00) for onset of standing heat with a vasectomized boar. At 16 and 24 hours after onset of standing heat (Day 0 of pregnancy), gilts were artificially inseminated using fresh, pooled, crossbred semen. Pregnant gilts were slaughtered on different days of pregnancy, and the reproductive tract was recovered within 10 minutes of death.

B. Collection of Endometrial and Embryonic Tissues

Uterine horns were excised at slaughter and individually flushed with physiological saline. Blastocysts were collected from the uterine flushings and rinsed once in a saline buffer. Spherical blastocsyts were sorted according to their size and

grouped as S1 (1-2 mm in diameter), S2 (5-6 mm) and S3 (8-10 mm). Elongated blastocysts, including ovoid, tubular and filamental stages, were pooled for individual pigs at different stages of pregnancy since they were frequently clumped together in the flushings. At the same time, sections of endometrial tissues were also harvested from the mid-section of each of two uterine horns for each individual pig. All the samples were immediately snap-frozen in liquid nitrogen after collection and stored at –80 °C.

C. Cell Culture

A trophoblast cell line, Jag-1, was originally isolated from trophoblastic tips of Day 14 porcine embryos, and was maintained as described (Ramsoondar et al., 1993). Porcine macrophages were isolated from "buffy coats" prepared from whole pig blood (Ramsoondar et al., 1993). Both porcine macrophages and Jag-1 cells were cultured in RPMI containing 10% (v/v) bovine calf serum and incubated at 37°C in an atmosphere of 5% (v/v) CO_2 in air. Two human small-cell lung cancer cell lines (H69B and H69C) (Dixon et al., 1995; Feyles et al., 1991) were included as negative controls for examination of CSF-1 expression, and were maintained in RPMI medium supplemented with 6% (v/v) bovine calf serum.

D. Cell Proliferation Bioassay

Jag-1 cells were cultured in SFM for 72 hr, and the conditioned medium was collected and concentrated about 10 times by filtration through a YM 10 (10k MW cut-off) membrane (Amicon, USA) within an ultrafiltration cell at 4 °C.

Proliferation bioassays of porcine macrophages and Jag-1 cells were carried out basically as described by Ramsoondar et al. (1993). Briefly, cells (5 x 10^4 cells/ well) in 100 µl of serum-free medium (SFM) (Gibco/BRL) were plated in each well of flat-

bottomed 96-well plates and were cultured for 2 hr. The medium in each well was replaced with 100 µl of test media and the cells were allowed to proliferate for a further 48 hr. The cells were subsequently pulsed by adding tritiated thymidine (1 µCi/well) in 20 µl SFM and further incubated for 18 hr. The cells were finally harvested and counted for tritiated thymidine incorporation using a Matrix 96 counter (Canberra Packard, USA).

A YYJ06 hybridoma cell line, producing anti-human CSF-1 monoclonal antibody, was kindly provided by Dr. L. Guilbert (University of Alberta).

E. RNA Extraction

Total cellular RNA was extracted using TRIzol reagent according to the manufacturer's instructions (Gibco/BRL). TRIzol reagent was directly added to the frozen embryonic tissues or cultured cells, whereas frozen endometrial tissues were first ground to a powder in a mortar and pestle in liquid nitrogen prior to addition of TRIzol reagent. RNA samples were dissolved in sterile distilled water and quantified by spectrophotometry.

F. Primer Design and RT-PCR

The coding sequences of human and mouse CSF-1 and c-fms were retrieved from the GenBank database (NCBI) and aligned and compared with the aid of a computer program, GeneJockey II (Biosoft, Cambridge, UK). Highly conserved regions between the two species, which are found at both ends of the CSF-1 coding sequence, were used to design primers for amplifying the cDNA sequence of porcine CSF-1 (Wong et al., 1987 and Ladner et al., 1988). The consensus sequences at the 3'-end of human and mouse c-fms coding sequences were used to generate primers for amplification of porcine

c-fms cDNA fragments (Rothwell and Rohrschneider, 1987; Coussens et al., 1986). DNA fragments from glyceraldehyde-3-phosphate dehydrogenase (G3PDH) and/or β-actin cDNA sequences were amplified as internal controls for gene expression using primers described previously (Yelich et al., 1997; Table 2.1). All primers and their sequences are shown in Table 2.1.

For amplification with the primer pair CSF-1U and CSF-1D, 1 μg of total RNA and 50 picomoles of the downstream primer (CSF-1D) were used for the reverse transcription (RT) reaction in a volume of 20 μl containing 50 mM Tris-HCl pH 8.3, 40 mM KCl, 5 mM $MgCl_2$, 0.5% Tween 20 (v/v), 1 mM dNTP, 10 mM DTT and 50 units Expand™ reverse transcriptase (Boehringer Mannheim). The RT reaction was carried out at 42°C for 1 hr, followed by incubation at 95 °C for 5 min. The RT product of 4 μl was then used directly for PCR in a 50-μl reaction volume containing 50 mM Tris-HCl pH 9.2, 160 mM $(NH_4)_2SO_4$, 1.75 mM $MgCl_2$, 350 μM dNTP, 300 nM each of CSF-1U and CSF-1D primers, and 2.5 units Expand™ long-template polymerase (Boehringer Mannheim). PCR parameters were denaturation at 94°C for 2 min, 30 cycles of (94 °C 10 sec, 65 °C 30 sec and 68 °C 2 min) and an additional 7-min extension at 68 °C.

For amplification of DNA fragments less than 1 kb in size, RT was basically carried out as described above, except oligo$(dT)_{15}$ primer was used instead of CSF-1D. PCR was carried out in a 50-μl reaction volume containing 4μl of RT products (only 0.1 μl for G3PDH and β-actin), 20 mM Tris HCl pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM dNTP, 200 nM each of upstream and downstream primers and 1.25 units *Taq* polymerase (Gibco/BRL).

G. Northern and Southern Blot Analysis

Probe DNA for both Northern and Southern blot hybridization was prepared using the DNA Random Primers Labeling Kit (Gibco/BRL) following the manufacturer's recommendations. Northern blots were prepared on nitrocellulose membranes (Micron Separations Inc., Massachusetts, USA). Total RNA was fractionated on a 1% (w/v) agarose gel containing 7% (v/v) formaldehyde in MOPS buffer. Northern hybridization was carried out at 65°C for 18 hr in 6x SSPE buffer containing 0.5% SDS and 5x Denhardt's solution. The blot was washed 3x 15 min in 2x SSC [0.1% (w/v) SDS] at room temperature and 1x 15 min in 0.1x SSPE [0.1% (w/v) SDS], and it was exposed to a Kodak XAR film for 48 hr at -80 °C.

Genomic DNA was isolated from Jag-1 cells as described (Ausubel et al., 1996), and digested with either *Bam* HI, *Eco* RI, *Hind* III or *Pst* I restriction endonucleases, respectively. Digests of 20 µg of DNA for each enzyme were fractionated on 0.8% (w/v) agarose gel in 1xTAE buffer at 2 V/cm for 10 hr. DNA capillary transfer was carried out in 0.4 M NaOH using Zeta-Probe membranes (Bio-Rad). Southern hybridization was carried out following the manufacturer's recommendations. Briefly, hybridization was carried out overnight at 43 °C in a solution containing 50% (v/v) formamide, 0.12 M Na_2HPO_4, 0.25 M NaCl, 7% (w/v) SDS, 1 mM EDTA, pH7.2. The blot was washed once 15 min at room temperature in both 2x SSC [0.1% (w/v) SDS] and 0.5x SSC [0.1% (w/v) SDS], respectively, and then once at 65 °C in 0.1x SSC [0.1% (w/v) SDS]. Exposure to a Kodak XAR film was done at -80 °C for 7 days.

RESULTS

A. Cell Proliferation Bioassay

The conditioned medium of Jag-1 cells was shown to stimulate the proliferation of porcine macrophages in a dose dependent fashion (Fig. 2.1A). This medium, when fractionated by reverse phase–FPLC, gave a single peak of activity with respect to the macrophage bioassay (data not shown). CSF-1 has been shown to be potent growth factor involved in the proliferation and differentiation of monocytic cells in both the human and the mouse (Stanley et al., 1983) and is therefore a potential candidate for the unknown porcine macrophage growth factor. Using a 1:1 (50%) dilution of the Jag-1 conditioned medium, an anti-human CSF-1 neutralizing monoclonal antibody YY106 was able to inhibit in a dose-dependent fashion the stimulatory activity in Jag-1 cell-conditioned medium (Fig. 2.1A). In addition, it was found in a parallel *in vitro* proliferation assay that YY106 could inhibit the proliferation of Jag-1 cells in a dose-dependent manner (Fig. 2.1B).

B. Cloning and Sequencing of Two Overlapping Fragments of a Porcine CSF-1 cDNA sequence

Using a RT-PCR approach, one predicted cDNA fragment of about 1.5 kb was amplified from Jag-1 total cellular RNA using the primer pair CSF-1U & CSF-1D (Fig. 2.2A), whereas no predicted fragment was amplified with another primer pair CSF-1S & CSF-1D (data not shown). The yield of the 1.5-kb amplicon from first round RT-PCR was low as indicated in Figure 2.2A and was insufficient for further analysis. Therefore, it was excised from the agarose gel using a clean razor blade and the gel slice was soaked

in 100 µl of distilled H_2O overnight at 4 °C. Sufficient yield was obtained after re-amplification with 5 µl of the elate using the same PCR conditions (Fig. 2.2B).

The 1.5-kb amplicon was cloned into the pCR-script Amp SK (+) vector (Stratagene) and was sequenced in both directions on an Automatic Sequencer (ABI/Prism 2.2.1). The resulting sequence shared significant homologies with human and mouse CSF-1 cDNA sequences. In order to obtain the missing 5'-end of the coding sequence for porcine CSF-1 gene, an internal primer, CSF-13 (Table 2.1 and Fig. 2.6), was designed according to the 5'-end sequence of the 1,569-nt cDNA fragment of porcine CSF-1 obtained above. RT was performed with the primer CSF-1D (Table 2.1 and Fig. 2.5). *Taq* polymerase (Gibco/BRL) was used for the subsequent PCR reaction with the primers CSF-1S and CSF-13 instead of Expand™ Long Template Polymerase used previously, because the predicted size of the PCR product from primers CSF-1S and CSF-13 was small. An amplicon with the expected size of 203 bp was obtained after RT-PCR. The identity of this amplicon was further confirmed by diagnostic restriction digestion with Pst I (Fig. 2.4) that has a restriction site at the 5'end of the 1,569-bp amplicon. The 203-bp amplicon was cloned and sequenced as described above. Therefore, the full-length coding sequence (1, 665 nt in length) of a porcine CSF-1 cDNA was obtained after alignment between the 1,569 nt and 203 nt overlapping partial sequences.

C. The Coding Sequence and Deduced Amino Acid Sequence of Porcine CSF-1

The full coding sequence of porcine CSF-1 was found to be 1,665 nt in length by assembling the sequences of the two overlapping amplicons of 1,569 bp and 203 bp, and encodes a protein precursor of 554aa with a 32aa peptide signal sequence (Fig. 2.5).

The coding sequence of porcine CSF-1 shares 82 % and 73 % sequence identity with human CSF-1 (Wong et al., 1987) and mouse CSF-1 (Ladner et al., 1988), respectively. The deduced amino acid sequence of porcine CSF-1 shares 73 % and 65 % homology with those of the human CSF-1 and the mouse CSF-1, respectively (Table 2.2). In addition, most of the structural features found in the predicted porcine CSF-1 precursor are common to those seen in the human and mouse proteins. Thirteen cysteines exist in the porcine CSF-1 precursor, twelve of which are conserved in the human and mouse proteins (Fig. 2.6). With the exception of the site at position N38, the other two sites of potential N-linked glycosylation at positions of N154 and N172 in porcine CSF-1 are conserved in human and mouse CSF-1 (Table 2.2). The glycosaminoglycan addition site is present as a short sequence "EEASGEASE" and is identical among the three CSF-1 precursors (Fig. 2.6). A highly hydrophobic domain from F495 to Y517 is followed by a short stretch of positively charged residues (R-R-R-R-W) and a cytoplasmic tail in the porcine CSF-1, which is typical of a transmembrane domain similar to those found in the human and mouse CSF-1 proteins (Fig. 2.6).

D. Expression of CSF-1 in Jag-1 Cells and Southern Blot Analysis of Porcine CSF-1 Gene

The cDNA fragment of 1,569 bp was used as a probe to examine the expression of the CSF-1 gene in cells of Jag-1, H69B and H69C cultured cells. Under the stringent washing conditions used (0.1x SSPE at 68°C), only one species of mRNA (about 4.0 kb) was detected in Jag-1 cells, and no hybridization signal was observed in either H69B or H69C human carcinoma cells, even after long exposure times (Fig. 2.3).

It was of interest to determine the number of copies of the CSF-1 gene in the porcine genome, since this information is needed for further studies on the structure and regulation of expression of the porcine CSF-1 gene. For this purpose, genomic DNA was extracted from Jag-1 cells and digested with four different restriction enzymes. Southern blot analysis was carried out using a radiolabeled porcine cDNA fragment of 1,569 bp as probe. One hybridizing fragment with approximate sizes of 2.2 kb, 23.0 kb and 25.0 kb was found in the *Bam* HI, *Eco* RI and *Hind* III digests, respectively, and two hybridizing fragments of about 1.3 kb and 4.6 kb in the *Pst* I digests (Fig. 2.7). These results suggest that there is only one copy of the CSF-1 gene in the pig genome.

E. Expression of CSF-1 transcripts in Endometrial and Embryonic Tissues during Pre-implantation Development

A major CSF-1 mRNA species of approximately 4 kb was detected by Northern blot analysis in RNA extracted from endometrial tissues at all pre-implantation developmental stages examined and in Jag-1 cells. Two minor mRNA species of about 5.1 kb and 3.6 kb, respectively, were also detected in endometrial tissues around Day 16 of pregnancy but not in Jag-1 cells (Fig. 2.3 and 2.8). Expression levels of the 4-kb CSF-1 mRNA in endometrial tissues of each of the pre-implantation developmental stages were normalized as a percentage of the 28S ribosomal RNA expression at their corresponding stages. High levels of CSF-1 mRNA expression were found in endometrial tissues at Day 3 of pregnancy, followed by a basal level of expression between Days 4 and 15.5 of pregnancy (Fig. 2.9). By Day 16 of pregnancy, CSF-1 mRNA level is elevated about 3 fold over basal levels in endometrial tissues.

CSF-1 mRNA levels in endometrial and embryonic tissues were compared at six different stages between Days 11 and 16 of pregnancy using RT-PCR with primers CSF-1S and CSF-13, since total RNA amounts obtained from embryos at earlier stages were limited. CSF-1 mRNA expression was up-regulated in embryonic tissues at the same period of time as in endometrial tissues with highest levels seen at Day 16. However, the absolute expression levels in embryonic tissues were much lower than in endometrial tissues at the six stages examined (Fig. 2.10).

F. Expression of c-fms transcripts in Embryonic and Endometrial Tissues during the Peri-implantation Period

A pair of primers, PR3 and PR6, was designed based on the 3'-end consensus regions of human and mouse c-fms coding sequences. A 303-bp cDNA fragment of porcine c-fms was amplified, cloned and sequenced from endometrial tissue RNA using the RT-PCR approach (Fig. 2.11). This fragment showed 85% and 79% sequence identity with the correspondent regions of human and mouse c-fms cDNA sequences. The deduced amino acid sequences shared 86% and 81% sequence identity with human and mouse sequences. Expression of c-fms mRNA was detected in endometrial tissues obtained between Days 9 and 15.75 of pregnancy using RT-PCR, but surprisingly not in blastocysts (Fig. 2.12A&D). Since we had already obtained strong evidence of a functionally signal pathway involving CSF-1 and, by definition, its receptor in embryo-derived trophoblast cells (Fig. 1), it was important to reconcile this discrepancy. Therefore, a pair of nested-primers CSF-1R7 and CSF-1R8, located within the 303-nt c-fms cDNA sequence characterized above, was needed to carry out a second round of PCR amplification (nest-PCR) after the initial RT-PCR with CSF-1R3 and CSF-1R6. The

identity of the nested-PCR product was confirmed by diagnostic digestion with a restriction enzyme *Alu* I, which was predicted to have a single cutting site within the 303-nt porcine sequence (Fig. 2.12F). Expression of c-fms mRNA was detected in higher levels in small blastocysts (1-2 mm in diameter) than in medium-sized blastocysts (5-6 mm), and progressively increased in both large blastocysts (8-10 mm) and elongated blastocysts obtained between Days 11 to 15.75 of pregnancy (Fig. 2.12E). Expression of c-fms mRNA therefore exhibits a similar pattern to its ligand, albeit at much lower levels, in blastocysts during peri-implantation development (Fig. 2.12C).

DISCUSSION

The stimulatory activity of conditioned media of Jag-1 cells, shown in the *in vitro* proliferation assay, suggests that Jag-1 cells could produce a soluble factor capable of stimulating the proliferation of porcine macrophages. This result is consistent with a previous report by Ramsoondar et al. (1993). This factor is present in conditioned serum-free medium harvested from Jag-1 trophoblast cells and as such represents a potential autocrine/ paracrine growth factor. The anti-human CSF-1 neutralizing monoclonal antibody YY106 can specifically inhibit the stimulatory activity in Jag-1 cell-conditioned medium, and also the proliferation of Jag-1 cells themselves, which provides strong evidence that CSF-1 is the bioactive growth factor produced by JAG-1 trophoblast cells. Taken together, these results suggest that porcine CSF-1 is an important aurocrine/paracrine growth factor for stimulating proliferation of pre-implantation trophoblast cells. These observations of a potential role for CSF-1 in regulating

proliferation of embryonic tissue(s) was the basis for fully characterizing the gene encoding porcine CSF-1 and its expression in embryonic and endometrial tissues.

Using a RT-PCR approach, a porcine CSF-1 cDNA has been cloned from Jag-1 cells derived from porcine embryos at Day 14 of pregnancy (Ramsoondar et al., 1993). The coding sequence of porcine CSF-1 shows an overall 82% and 73% sequence identity with those of human and mouse CSF-1, respectively, at the nucleotide level. In an earlier study (Tuo et al., 1995), a partial coding sequence (823 nt) of porcine CSF-1 was obtained from Day 15 porcine embryos, and was reported to have greater than 98% identity with the human counterpart (sequence data was not published or submitted to the GenBank database). However, the corresponding 823-nt sequence characterized in the present study shows 87% sequence identity to the human counterpart. The reason for this discrepancy is unknown.

Using the porcine CSF-1 cDNA clone as a probe, one major mRNA species of about 4 kb was detected in Jag-1 cells and all endometrial tissues examined by Northern blot analysis. In addition, two extra mRNA species of about 5.1 kb and 3.6 kb, respectively, were also detected in endometrial tissues around Day 16 of pregnancy but not in Jag-1 cells. These results are consistent with the findings in a previous study (Tuo et al., 1995), where the two mRNA species of 5.1 kb and 3.6 kb were detected in porcine endometrial and oviductal tissues at Day 15 but not in embryonic tissues at Day 15 by Northern hybridization analysis, using a human CSF-1 cDNA probe. Similar transcript size heterogeneity occurs in many types of human tissues and cells, including pregnant endometrium and placenta, monocytes, endothelial cells, fibroblasts and keratinocytes, where the 4-kb CSF-1 mRNA species was also found to be predominantly expressed

(Daiter et al., 1992; Stanley, 1990; Seelentag et al., 1987; Akashi et al., 1989; Chodakewitz et al., 1990). In the mouse, the predominant CSF-1 mRNA species was found to be 2.3 kb in length in pregnant uterus, although it was not detectable in nongravid uterus, in which the 4-kb species was predominantly expressed (Arceci et al., 1989).

The present study demonstrates that porcine endometrial CSF-1 mRNA expression reaches a peak on Day 3 of pregnancy, when embryos are still in the oviduct. It is known that following insemination the majority of sperm remain in the uterus on their way towards the fertilization site in the oviduct. CSF-1 has been shown to act as a chemotactic factor for monocytes and to promote the proliferation and differentiation of macrophages (Sherr and Stanley, 1990; Stanley, 1990). Observations in rodents suggest that variations in the uterine macrophage population might be an indirect effect of the release of CSF-1 by the glandular epithelium (De and Wood, 1990; Pollard et al., 1991). In addition, it has been demonstrated in the mouse that the acute accumulation of uterine macrophages on Days 1 and 2 of pregnancy occurs coincident with peak expression of CSF-1 in the uterus, which functions primarily in the elimination of sperm from the uterus (Wood et al., 1997). Therefore, peak CSF-1 expression around Day 3 in the pig might have its primary function in recruiting monocytes and promoting proliferation and differentiation of uterine macrophages in order to eliminate the large number of sperm remaining in the uterine lumen. Thereafter, an appropriate uterine environment could be established for subsequent embryo development in the uterus.

In this study, porcine endometrial CSF-1 mRNA expression level was elevated by about 3-fold on Day 16 in comparison to basal levels observed between Days 4 and

15.5. CSF-1 mRNA levels were also up regulated around Day 16 of pregnancy in embryonic tissues, although at much lower absolute levels. The lower levels of embryo-derived CSF-1 in samples obtained from pigs between Days 9 and 15.75 of pregnancy indicate that the endometrium might be a more important source of CSF-1 during pre-implantation development of porcine embryos. In contrast, the allantochorionic placenta was found to express a higher level of CSF-1 than the endometrium during the post-implantation period in the pig (Tuo et al., 1995). More importantly, mRNA expression for both CSF-1 and c-fms were detected and exhibited similar temporal patterns in porcine embryos during peri-implantation development. In addition, CSF-1 was shown to be the major autocrine factor regulating the proliferation of trophoblast cells *in vitro* in the present study. Taken together, these results suggest that CSF-1 might act in both autocrine and paracrine fashion on the proliferation of porcine trophoblast cells during the pre-implantation period, which is in contrast to only a paracine role of CSF-1 on mouse pre-implantation embryos (Arceci et al., 1992).

Over-expression of c-fms was shown to facilitate cellular invasion and anchorage-independent growth in mammary epithelial cells, and to reduce the concentration of retinoic acid or 1,25-dihydroxy vitamin D3 needed to cause monocytic cell differentiation (Sapi et al., 1996; Yen et al., 1997). In addition, c-fms was shown to be expressed at very high levels on placental trophoblasts, especially invasive trophoblast (reviewed by Hume et al., 1997; Aboagye-Mathiesen et al., 1997; Pampfer et al., 1992). However, expression of c-fms mRNA was observed at very low levels in pre-implantation porcine blastocysts in the present study. Therefore, we could speculate that the expression level of c-fms in porcine trophoblast cells might be not enough to induce

the differentiation of trophoblast cells that is essential for the acquisition of invasiveness by trophoblast cells during embryo implantation as seen in humans and mice. As a result, trophoblast cells could not acquire the ability *in vivo* to invade the endometrium during embryo implantation in pigs, although they appeared to be invasive after they were transferred to ectopic sites (Samuel, 1971 & 1972; Samuel and Perry, 1972).

Our Southern blot analysis indicates that the porcine CSF-1 gene presents as a single copy in the genome. In conjunction with previous studies, our results suggest that porcine CSF-1 might be very similar to human and mouse CSF-1 in gene structure and its expression regulation during pregnancy. The present study will certainly facilitate further studies on the structure and expression regulation of the porcine CSF-1 gene and on its potential roles in porcine implantation and placental development.

It should be noted that the last 18 nucleotides of the coding sequence of porcine CSF-1 are not completely derived from porcine CSF-1 mRNA, since the down-stream primer CSF-1D is designed from the conserved region between human and mouse sequences. However, we may assume that it is very similar between pig, human and mouse sequences in this region. To confirm the functionality of the cloned porcine CSF-1 cDNA sequence, we have introduced the full-length coding sequence of porcine CSF-1 into the genome of COS-7 cells through an eukaryotic expression vector and are currently evaluating the activity of the CSF-1 in a sensitive bioassay.

Table 2.1 Sequences of CSF-1 and c-fms primers designed in this experiment [a]

Primer	Sequence [b] (Shown as 5' → 3')	Annealing temperature used for PCR amplification
CSF-1S	TGAATTCCCAGCTGCCCCGTATG	58 °C
CSF-15	TGCCCCGTATGACCGCGCCG	
CSF-13	GTCTCCATCTGACTGTCAATCAGCTG	
CSF-1U	TGAATTCACTGTAGCCACATGATTGGG	65 °C
CSF-1D	TAGATCTCTACACTGGCAGTTCCAC	
PR3	CTCAGCAGAACTGATAGTTGTTGGGCTGC	65 °C
PR6	AACTGGTGAAGGATGGATACCAAATGGC	
PR7	TACAGCATCATGCAGGCCTGCTGGG	60 °C
PR8	ATCCCCCTGCTCGCAGCAGGCCA	
G3PDH-U	ACCACAGTCCATGCCATCAC	60 °C
G3PDH-D	TCCACCACCCTGTTGCTGTA	
Actin-U	ATCTTGATCTTCATGGTGCTGGGC	
Actin-D	ACCACTGGCATTGTCATGGACTCT	

[a] Locations and directions of primers are shown in Figure 5 and Figure 12.

[b] A restriction site (underlined) is attached to some of the primers.

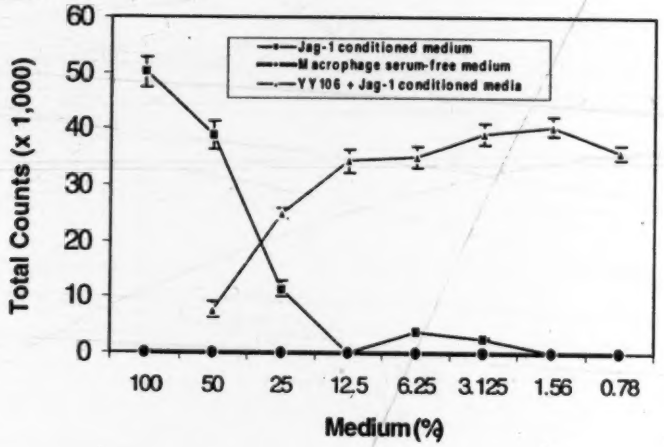
Table 2.2 The homologies of the coding sequences and deduced amino acid sequences of porcine, human and mouse CSF-1[a], and the positions of potential N-linked glycosylation (N-X-S/T) and glycosaminoglycan addition (acidic residues-S-G-X-G/A)

	Homology[b] of the coding sequence (deduced **amino acid sequence**)			Positions[c] of N-X-S/T	Positions of glycosaminogly-can addition site
	H CSF-1	M CSF-1	P CSF-1		
P CSF-1	82% (**73%**)			38;**154**;**172**	309
H CSF-1		77% (**67%**)		**154**;**172**;381;415	309
M CSF-1			73% (**65%**)	107;**154**,**172**,378	308

[a] Rat CSF-1 is not included because its coding sequence is identical to that of mouse CSF-1 (Borycki et al., 1993), and the short forms (256aa and 438aa) of human CSF-1 (Kawasaki et al., 1985) are also not included here. [b] The data were obtained with the aid of a computer program (GeneJockey II). [c] Amino acids were counted by referring the position of starting methionine as the first one.

Figure 2.1 A). Stimulatory effect (■) of Jag-1 cell-conditioned medium (CM) and inhibitory effect (▲) of YY106 hybridoma CM on the proliferation of porcine monocyte/macrophages. Peak stimulation occurred at 100% Jag-1 CM. The stimulatory effect in 50% Jag-1 CM was blocked in a dose-dependent fashion by YY106 hybridoma CM. Macrophage serum-free medium was used as a control (●) in this experiment. B). Inhibitory effect of YY106 hybridoma CM on the proliferation of Jag-1 cells was in a dose-dependent fashion. Results were presented as the mean ± SEM of six reaction replicates.

A


Jag-1 conditioned medium
Macrophage serum-free medium
YY106 + Jag-1 conditioned media
Total Counts (x 1,000)
60
50
40
30
20
10
0
100
50
25
12.5
6.25
3.125
1.56
0.78
Medium (%)

B

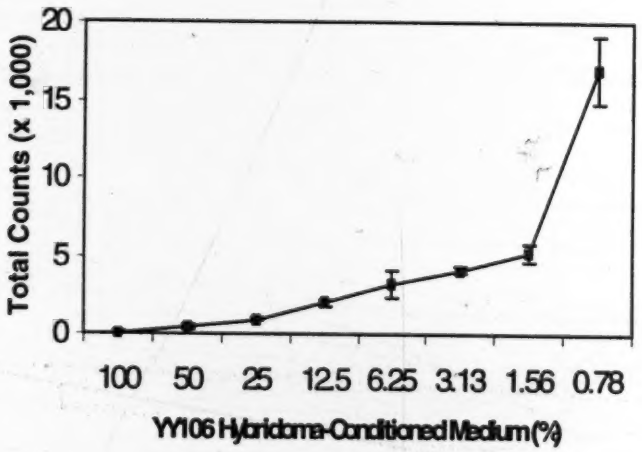
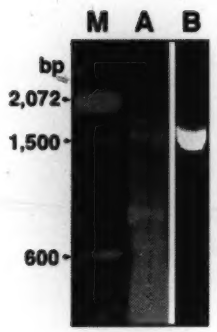

Total Counts (x 1,000)
20
15
10
5
0
100
50
25
12.5
6.25
3.13
1.56
0.78
YY106 Hybridoma-Conditioned Medium (%)

Figure 2.2 (Top) The amplification of the 1,569-bp cDNA fragment from mRNA of Jag-1 cells. Lane A, one fifth of the products from RT-PCR, which was carried out using total RNA (1μg) of Jag-1 cells and the primer pair CSF-1U & CSF-1D, was fractionated in 0.8% (w/v) agarose gel. Lane B, the amplicon of 1,569 bp was isolated and re-amplified under the same conditions and with the same primer pair as in RT-PCR. One fifth of the PCR product was run in 0.8% (w/v) agarose gel. Both of the gels were stained with ethidium bromide. Lane M, 100-bp DNA ladder of 0.5 μg.

Figure 2.3 (Bottom left) Northern blot hybridization analysis of RNA extracted from Jag-1, H69B and H69C cells. The 1,569-bp cDNA fragment of porcine CSF-1 gene was used as the probe. Twenty micrograms of total RNA from Jag-1 (Lane 1), H69B (Lane 2) and H69C (Lane 3) cells was denatured and fractionated on a 1% (w/v) agarose gel containing 7% (v/v) formaldehyde in MOPS buffer.

Figure 2.4 (Bottom right) RT-PCR and restriction analysis of the 5' end region of porcine CSF-1 coding sequence. RT was done under the same conditions as described in Fig. 2.2, and PCR was carried out with the primer pair CSF-1S & CSF-13. Electrophoresis was done in 1.3% (w/v) agarose gel, and the gel was stained in ethidium bromide. Lane M, 100-bp DNA ladder. Lane A, RT-PCR products of 8 μl. Lane B, restriction digests of the RT-PCR products of 10 μl with *Pst* I (37°C, 1 hr).


M A B
bp
2,072
1,500
600

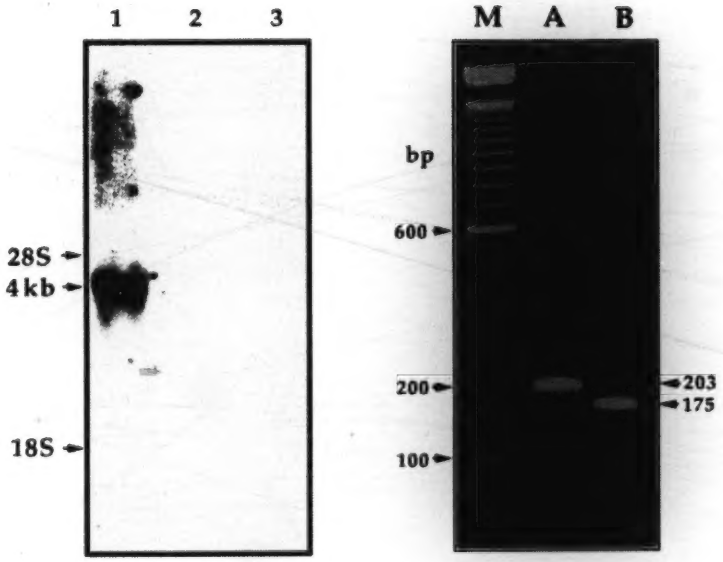
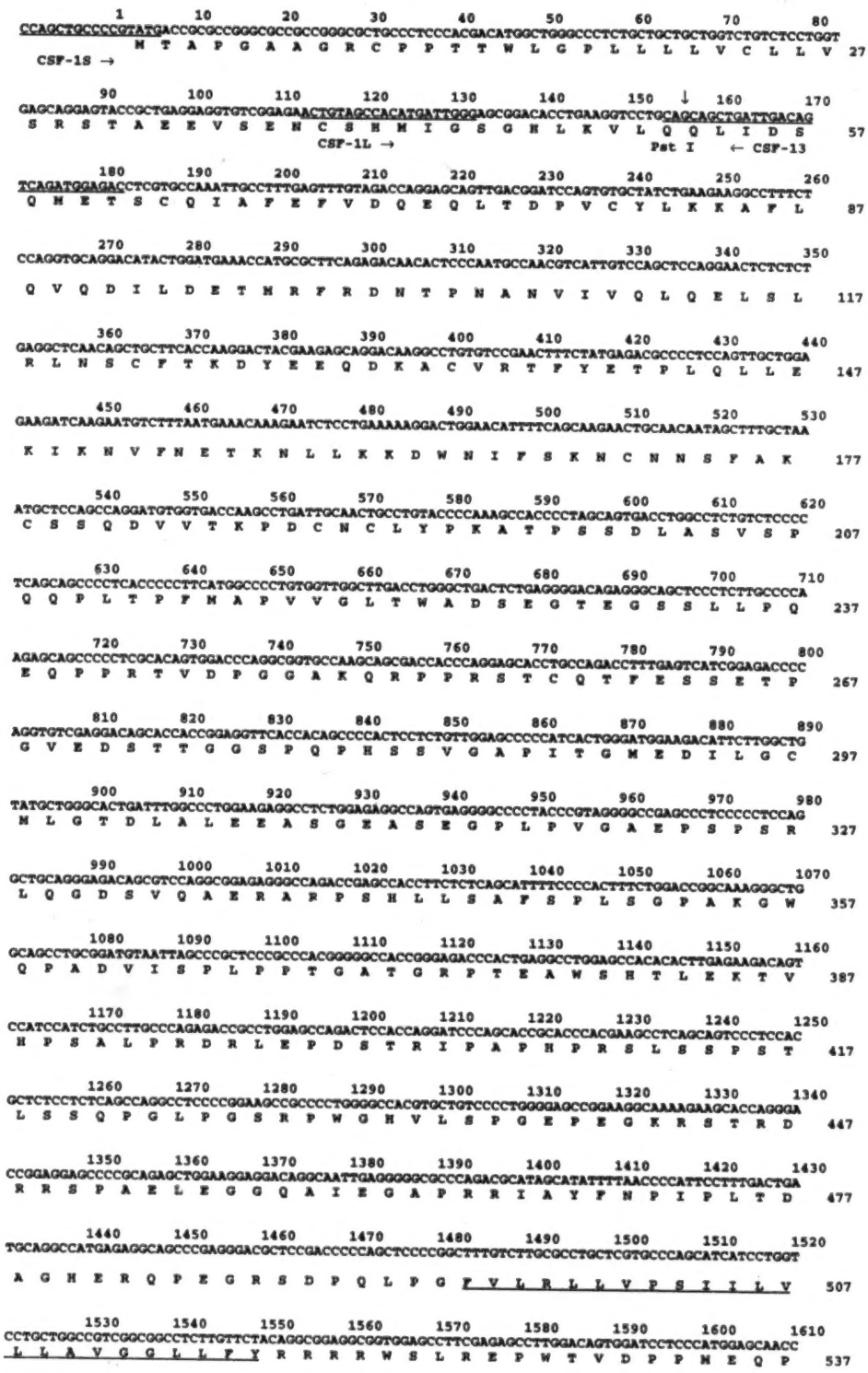

1 2 3
28S
4kb
18S
M A B
bp
600
200
100
203
175

Figure 2.5 Nucleotide sequence and deduced amino acid sequence of porcine CSF-1. The numberings of the nucleotides and amino acids begin at the start methionine code and are indicated above and to the right of their corresponding sequences, respectively. The positions of the primers used for PCR in this study are underlined under the nucleotide sequence, with arrows indicating their priming directions. The transmembrane domain is underlined at the bottom of the amino acid sequence. The *Pst* I restriction site at the 5' end is indicated with a vertical arrow.


CSF-1S →
CSF-1L →
Pst I
← CSF-13

1620 1630 1640 1650 1660
AGAGGGCAGCCCCCTGACCCAGGATGAGGACAGACAGGTGGAACTGCCAGTGTAG
E G S P L T Q D E D R Q V E L P V * 554
← CSF-1R

Figure 2.6 Comparison of the amino acid sequences of porcine, human and mouse CSF-1. The sequence alignment was done with the GeneJockey II computer program. The bullet mark (•) indicates a perfect agreement between sequences. Potential N-linked glycosylation sites are underlined with solid lines, and the glycosaminoglycan addition site is underlined with a dotted line. The transmembrane domain is indicated with a double dotted line.

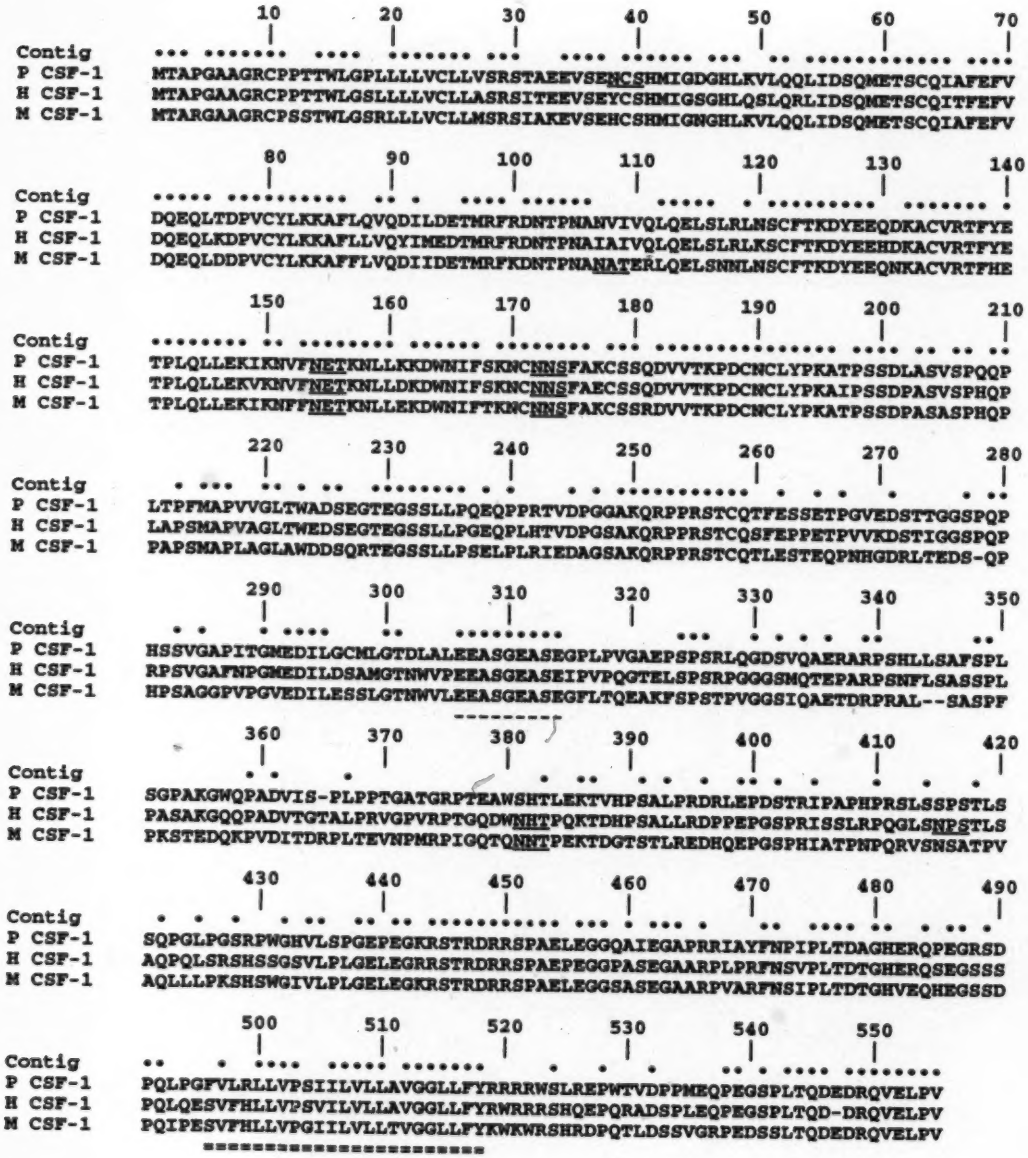

10 20 30 40 50 60 70
Contig
P CSF-1 MTAPGAAGRCPPTTWLGPLLLLVCLLVSRSTAEEVSENCSHMIGDGHLKVLQQLIDSQMETSCQIAFEFV
H CSF-1 MTAPGAAGRCPPTTWLGSLLLLVCLLASRSITEEVSEYCSHMIGSGHLQSLQRLIDSQMETSCQITFEFV
M CSF-1 MTARGAAGRCPSSTWLGSRLLLVCLLMSRSIAKEVSEHCSHMIGNGHLKVLQQLIDSQMETSCQIAFEFV
80 90 100 110 120 130 140
Contig
P CSF-1 DQEQLTDPVCYLKKAFLQVQDILDETMRFRDNTPNANVIVQLQELSLRLNSCFTKDYEEQDKACVRTFYE
H CSF-1 DQEQLKDPVCYLKKAFLLVQYIMEDTMRFRDNTPNAIAIVQLQELSLRLKSCFTKDYEEHDKACVRTFYE
M CSF-1 DQEQLDDPVCYLKKAFFLVQDIIDETMRFKDNTPNANATERLQELSNNLNSCFTKDYEEQNKACVRTFHE
150 160 170 180 190 200 210
Contig
P CSF-1 TPLQLLEKIKNVFNETKNLLKKDWNIFSKNCNNSFAKCSSQDVVTKPDCNCLYPKATPSSDLASVSPQQP
H CSF-1 TPLQLLEKVKNVFNETKNLLDKDWNIFSKNCNNSFAECSSQDVVTKPDCNCLYPKAIPSSDPASVSPHQP
M CSF-1 TPLQLLEKIKNFFNETKNLLEKDWNIFTKNCNNSFAKCSSRDVVTKPDCNCLYPKATPSSDPASASPHQP
220 230 240 250 260 270 280
Contig
P CSF-1 LTPFMAPVVGLTWADSEGTEGSSLLPQEQPPRTVDPGGAKQRPPRSTCQTFESSETPGVEDSTTGGSPQP
H CSF-1 LAPSMAPVAGLTWEDSEGTEGSSLLPGEQPLHTVDPGSAKQRPPRSTCQSFEPPETPVVKDSTIGGSPQP
M CSF-1 PAPSMAPLAGLAWDDSQRTEGSSLLPSELPLRIEDAGSAKQRPPRSTCQTLESTEQPNHGDRLTEDS-QP
290 300 310 320 330 340 350
Contig
P CSF-1 HSSVGAPITGMEDILGCMLGTDLALEEASGEASEGPLPVGAEPSPSRLQGDSVQAERARPSHLLSAFSPL
H CSF-1 RPSVGAFNPGMEDILDSAMGTNWVPEEASGEASEIPVPQGTELSPSRPGGGSMQTEPARPSNFLSASSPL
M CSF-1 HPSAGGPVPGVEDILESSLGTNWVLEEASGEASEGFLTQEAKFSPSTPVGGSIQAETDRPRAL--SASPF
360 370 380 390 400 410 420
Contig
P CSF-1 SGPAKGWQPADVIS-PLPPTGATGRPTEAWSHTLEKTVHPSALPRDRLEPDSTRIPAPHPRSLSSPSTLS
H CSF-1 PASAKGQQPADVTGTALPRVGPVRPTGQDWNHTPQKTDHPSALLRDPPEPGSPRISSLRPQGLSNPSTLS
M CSF-1 PKSTEDQKPVDITDRPLTEVNPMRPIGQTQNNTPEKTDGTSTLREDHQEPGSPHIATPNPQRVSNSATPV
430 440 450 460 470 480 490
Contig
P CSF-1 SQPGLPGSRPWGHVLSPGEPEGKRSTRDRRSPAELEGGQAIEGAPRRIAYFNPIPLTDAGHERQPEGRSD
H CSF-1 AQPQLSRSHSSGSVLPLGELEGRRSTRDRRSPAEPEGGPASEGAARPLPRFNSVPLTDTGHERQSEGSSS
M CSF-1 AQLLLPKSHSWGIVLPLGELEGKRSTRDRRSPAELEGGSASEGAARPVARFNSIPLTDTGHVEQHEGSSD
500 510 520 530 540 550
Contig
P CSF-1 PQLPGFVLRLLVPSIILVLLAVGGLLFYRRRRWSLREPWTVDPPMEQPEGSPLTQDEDRQVELPV
H CSF-1 PQLQESVFHLLVPSVILVLLAVGGLLFYRWRRRSHQEPQRADSPLEQPEGSPLTQD-DRQVELPV
M CSF-1 PQIPESVFHLLVPGIILVLLTVGGLLFYKWKWRSHRDPQTLDSSVGRPEDSSLTQDEDRQVELPV

Figure 2.7 Southern blot hybridization analysis of Jag-1 cell DNA digested with restriction enzymes, *Bam* HI (Lane A), *Eco* RI (Lane B), *Hind* III (Lane C) and *Pst* I (Lane D), respectively. The blot was hybridized with the same probe DNA as used for Northern blot hybridization (Fig. 2.3), except that the labeled probe DNA was separated from unincorporated α-^{32}p-dATP before adding to the hybridization buffer.

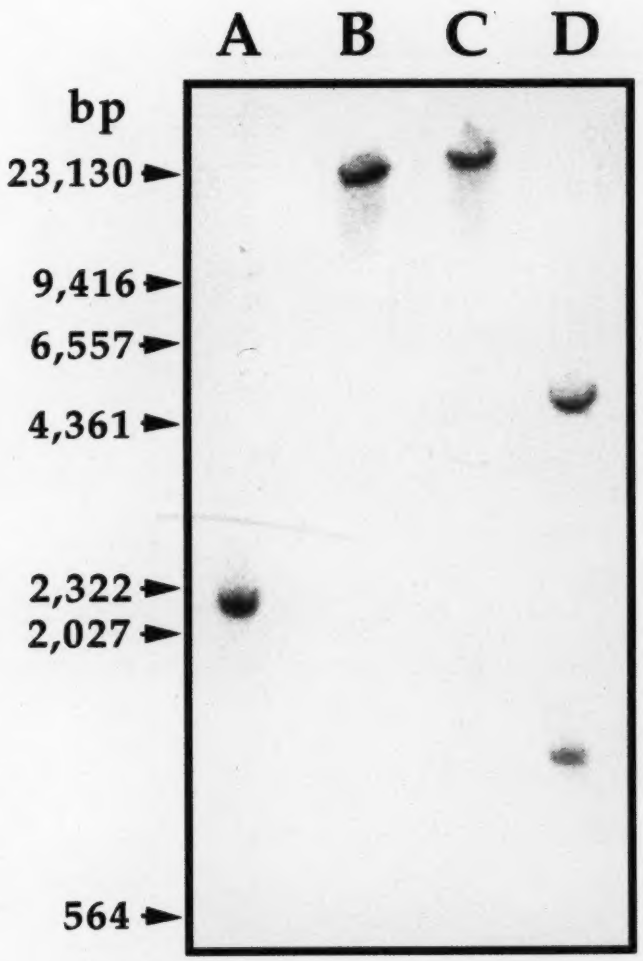
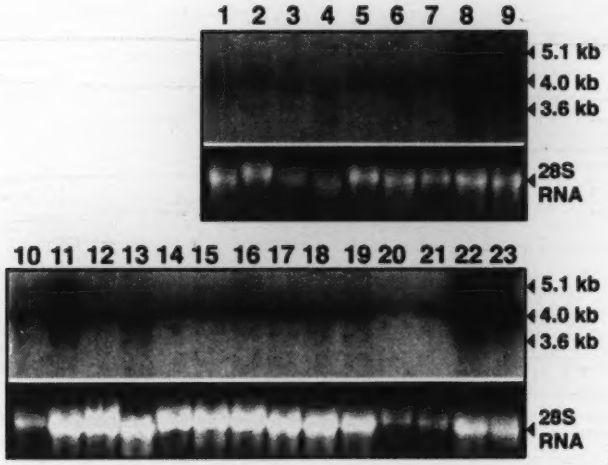

A
B
C
D
bp
23,130
9,416
6,557
4,361
2,322
2,027
564

Figure 2.8 (Upper two panels) Northern hybridization analysis of RNA extracted from endometrial tissues during pre-implantation development. Lanes 1 to 22 represent endometrial RNA samples from 21 individual pigs on Days 9.5, 11, 11.75, 12, 12, 13.5, 15.5, 15.75, 15.75, 3, 3, 4, 5.5, 5.5, 7, 7.5, 9, 9.5, 10.75, 12.75, 13.75, 16.5 of pregnancy, respectively. Samples in Lanes 1 and 19 are from the same Day 9.5 pig. Lane 23 is RNA from Jag-1 cells which was used as a positive control. In the upper panel are Northern hybridization signals, and in the lower panel are the corresponding 28S RNA amounts on the nitrocellulose membrane after transfer from the agarose gel, which are used as the internal control of RNA loading for quantitative analysis. The 1,569-bp cDNA fragment of porcine CSF-1 gene was used as the probe.

Figure 2.9 (Lower panel) Quantitative analysis of the 4-kb CSF-1 mRNA expression in endometrial tissues between Day 3 and Day 16.5. Data for CSF-1 mRNA levels and 28S RNA levels were obtained using the Imaging Densitometer GS-670 (BioRad) and the Gel Doc 1000 (BioRad) with the aid of the Molecular Analyst Software (Version 2.1) (BioRad, California). Expression levels of the 4-kb CSF-1 mRNA in each of endometrial samples were normalized as percentages of their corresponding 28S RNA levels on the nitrocellulose membranes.


1 2 3 4 5 6 7 8 9
5.1 kb
4.0 kb
3.6 kb
28S RNA
10 11 12 13 14 15 16 17 18 19 20 21 22 23
5.1 kb
4.0 kb
3.6 kb
28S RNA

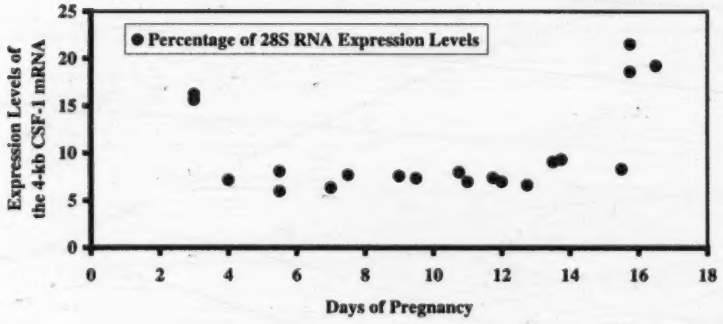

Percentage of 28S RNA Expression Levels
Expression Levels of the 4-kb CSF-1 mRNA
Days of Pregnancy
0
5
10
15
20
25
0
2
4
6
8
10
12
14
16
18

Figure 2.10 Comparison of CSF-1 mRNA expression levels between endometrial and embryonic tissues during pre-implantation development. Lanes 1 to 7 represent 7 individual pigs at Days 11, 11.75, 12, 13.5, 15.5; 15.75 and 15.75 of pregnancy, respectively. Lane T, Jag-1 cells. Both endometrial and embryonic RNA were isolated from each of these pigs. Using RT-PCR, primers CSF-15 and CSF-13 were used to amplify the 5' end 197-bp sequence of porcine CSF-1 including the deduced signal sequence, which is common to all cDNA sequences of human CSF-1 identified so far. CSF-1 PCR products were confirmed by restriction digestion with Pst I as in Fig. 4. RNA from Jag-1 cells was used as a positive control, and β-actin and G3PDH were co-amplified in all RNA samples as internal controls of gene expression.

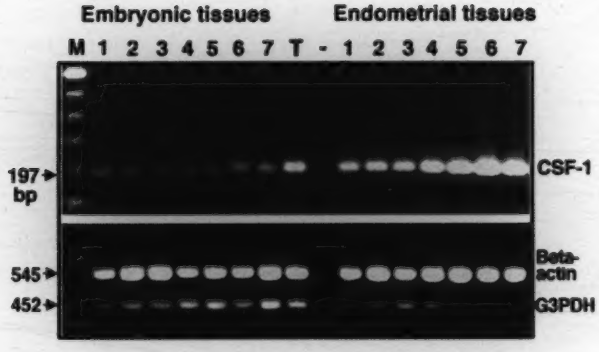

Embryonic tissues
Endometrial tissues
M 1 2 3 4 5 6 7 T - 1 2 3 4 5 6 7
197 bp
CSF-1
545
452
Beta-actin
G3PDH

Figure 2.11 A). Nucleotide sequence of the 303-bp fragment of porcine c-fms cDNA. The primer sequences and directions are indicated with underlines and arrows, respectively. The termination code is boxed. B). Comparison of the deduced 112 amino acid sequences of porcine, human and mouse c-fms. The bullet mark (•) indicates a perfect agreement between sequences. The variable region between sequences is indicated within two vertical slashes.

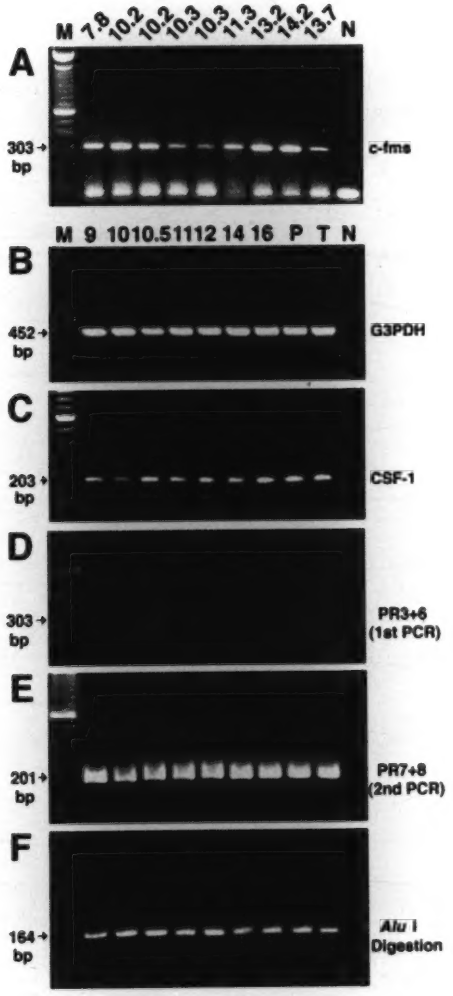
A

```
                          PR6→
AACTGGTGAA GGATGGATAC CAAATGGCCC AGCTGGCGTT CGCCCCCAAG AACATATACA
                     PR7→
GCATCATGCA GGCCTGCTGG GCCCTGGAGC CGACGCGCAG ACCCACCTTC CAGCAGATCT

GCTCCCTCCT TCAGGAGCAG AGCCACGGCG ACCAGAGAGC GCGGGACTAC AGCAACCTGC
                                      AluI↓                ←PR8
CGAGCAGCAG CAGCAGCAGC AGCGAGCCCG AGGAGGGGAG CTCCGGCGAG CACCTGGCCT
                                     ←PR3
GCTGCGAGCA GGGGGATGCG GCCCAGCCCC TGCTGCAGCC CAACAACTAT CAGTTCTGCT GAG
```

B

```
                 10        20        30        40        50
                  |         |         |         |         |
Contig    •••••••••• ••••••••• •• •••• ••••••••  ••••     •  •• ••
Human     LVKDGYQMAQPAFAPKNIYSIMQACWALEPTHRPTFQQICSFLQEQAQEDRRERDYTNL
Mouse     LVKDGYQMAQPVFAPKNIYSIMQSCWDLEPTRRPTFQQICFLLQEQARLERRDQDYANL
Pig       LVKDGYQMAQLAFAPKNIYSIMQACWALEPTRRPTFQQICSLLQEQSHGDQRARDYSNL

          60        70        80        90       100       110
          |         |         |         |         |         |
Contig    •••|             |•••• •• •• ••• ••• •• ••••••••••••
Human     PSSSRSGGS-------GSSSSELEEESSSEHLTCCEQGDIAQPLLQPNNYQFC•
Mouse     PSSGGSSGSDSGGGSSGGSSSEPEEESSSEHLACCEPGDIAQPLLQPNNYQFC•
Pig       PSSS-------------SSSSEPEEGSSGEHLACCEQGDAAQPLLQPNNYQFC•
```

Figure 2.12 A). RT-PCR amplification (45 cycles) of c-fms mRNA in porcine endometrium. Lanes 1-9 represent endometrial RNA samples obtained from individual pigs on Day 9.5, 11, 11.75, 12, 12, 13.5, 15.5, 15.75 and 15.75 of pregnancy, respectively. B-F). PCR amplification of CSF-1 and c-fm mRNA in porcine pre-implantation blastocysts [RT-PCR of G3PDH (B), CSF-1 (C), and c-fms (D); Nest-PCR of c-fms (E); Diagnostic digestion of nest-PCR products in gel E with *Alu* I (F)]. M, 100-bp DNA ladder. Samples in gels B-F were spherical blastocysts of 1-2 mm (Lane 9), 5-6 mm (Lane 10) and 8-10 mm (Lane 10.5) in diameter and elongated blastocysts obtained on Day 11 (Lane 11), 12 (Lane 12), 14 (Lane 14) and 16 (Lane 16) of pregnancy, respectively. Electrophoresis was carried out in 1.2 % agarose gel, and gels were stained with ethidium bromide.


M 7.8 10.2 10.2 10.3 10.3 11.3 13.2 14.2 13.7 N
A
303 → bp
c-fms
M 9 10 10.5 11 12 14 16 P T N
B
452 → bp
G3PDH
C
203 → bp
CSF-1
D
303 → bp
PR3+6 (1st PCR)
E
201 → bp
PR7+8 (2nd PCR)
F
164 → bp
Alu I Digestion

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CHAPTER 3

EXPRESSION OF MATRIX METALLOPROTEINASES AND THEIR REGULATORS DURING PORCINE PERI-IMPLANTATION DEVELOPMENT

INTRODUCTION

Unlike rodent and human blastocysts that start to attach to, and penetrate into, the endometrium immediately after hatching, porcine blastocysts undergo a prolonged pre-implantation development period involving an initial expansion, followed by appropriate spacing and conceptus elongation and subsequent attachment in the uterus (Flood, 1991). Upon expanding to a diameter of about 10 mm, porcine blastocysts elongate dramatically from a spherical to a filamentous form that can be up to 100 cm in length. This elongation precedes the physical attachment which occurs between the trophoblast and the uterine epithelium (Geisert et al., 1982a). During initial elongation from Day 10 to 12 of pregnancy (Day O designated as onset of estrus), the physiological activities of the blastocyst increase, as evidenced by the onset of estrogen (Gadsby et al., 1980; Fischer et al., 1985) and protein synthesis (Godkin et al., 1982). The onset of embryonic estrogen synthesis is followed by a significant increase in intraluminal calcium content and a rapid release of secretory vesicles from uterine epithelial cells into the lumen (Geisert et al., 1982b; Stroband & Van der Lende, 1990). Along with the elongation process, the extra-embryonic mesoderm initiates development and migrates between the trophoblast and the extraembryonic endoderm (Patten, 1948). The trophoblast and the uterine epithelium become closely apposed to each other immediately after the blastocyst elongation is complete (Flood, 1991). This is followed by adhesion between these two cell layers around Day 14 of pregnancy, as seen by microvillar

interdigitation between the two epithelial layers by Day 16 of pregnancy (Dantzer, 1985; Keys and King, 1990).

Implantation of the blastocyst is an essential step during mammalian pregnancy, leading to formation of a placenta capable of supporting embryonic/fetal development to term. The endocrine milieu of the mammal, and in particular its progesterone and estrogen status, is essential for establishing appropriate uterine conditions for implantation (de Ziegler et al., 1998; Ace and Okulicz, 1995; Bazer et al., 1982). The processes leading to early embryonic development and implantation also involve non-hormonal factors, which include the expression of a variety of adhesion molecules, vasoactive agents, tissue remodeling enzymes and their regulators, as well as cytokines/ growth factors. Matrix metalloproteinases (MMP) or matrixins are a group of at least 16 Zn^{2+} endopeptidases, which can degrade extracellular matrix (ECM) components such as collagen(s), laminin, fibronectin and vitronectin (Parsons et al. 1997; Nagase, 1997; Benbow and Brinckerhoff, 1997). Most of the MMPs are secreted as zymogens (proMMPs), and as an example activation of proMMP-2 at the cell surface can be mediated *in vivo* by membrane type-1 MMP (MMP-14 or Mt1-MMP) that is abundantly expressed in placenta (Parsons et al., 1997; Corcoran et al., 1996; Will et al., 1995). Zymogens of other MMPs can be activated through the action of plasmin by urokinase-type plasminogen activator (uPA) (Parsons et al., 1997). MMPs play a major role in tissue remodeling in a variety of physiological processes, which include embryo development, morphogenesis, angiogenesis and tissue involution, as well as pathological processes such as tissue ulceration, arthritis and cancers (Damsky et al., 1997; Woessner, 1994). MMPs have also been implicated in the subtle modulation of cell-matrix

interactions governing processes as diverse as cellular differentiation and migration (Basbaum and Werb, 1996). Tissue inhibitors of metalloproteinases (TIMPs) are the specific inhibitors for MMPs, and there are four members characterized in this family. TIMP-1 and TIMP-2 are present in a soluble form and play a key role in maintaining the balance between ECM deposition and degradation in different physiological processes (Gomez et al., 1997). TIMP-3 is insoluble, and is the only member of the TIMP family that is found exclusively in the ECM (Leco et al., 1994). TIMP-4 is mainly expressed in the adult heart with low levels in the placenta in the human, and is mainly expressed in adult brain and heart in the mouse (Leco et al., 1997; Greene et al., 1996). The role of MMPs and TIMPs during embryo implantation has been extensively studied in the mouse and human (Damsky et al., 1997; Bass et al., 1997; Alexander et al., 1996). In the pig, a previous study on the localization and expression of MMPs and TIMPs at the time of embryo-uterine contact was carried out by Menino et al. (1997).

Embryo implantation during pregnancy in pigs is a non-invasive process that results in simple attachment between the trophoblast and the uterine epithelium. This type of placentation is termed epitheliochorial to distinguish it from other more invasive types of placentation, such as the hemochorial placentation seen in rodents and humans. Strikingly, when porcine embryos were artificially transplanted to ectopic sites, the trophoblast cells appeared to be invasive (Samuel, 1971and 1972; Samuel and Perry, 1972). This suggests that either the environmental cues present at the ectopic site caused these cells to become invasive or, alternatively, that the intrinsically invasive nature of these cells must be regulated *in vivo* during normal conceptus development. If the latter is correct, then the balance between ECM degradation and deposition in the embryo, in the

endometrium, and at the interface between the trophoblast and the uterine epithelium must be tightly controlled, during the peri-implantation period, such that implantation and early embryogenesis can proceed appropriately. The objective of this study was to characterize the temporal expression of MMPs and their regulators in blastocysts and the endometrium during the peri-implantation period in the pig.

MATERIALS AND METHODS

A. Animals

Sexually mature gilts (PIC Camborough) of similar age and weight, with two recorded estrous cycles, were heat-checked three times daily (08:00, 16:00 and 00:00) for onset of standing heat with a vasectomized boar. At 16 and 24 hours after onset of standing heat, gilts were artificially inseminated using fresh-pooled crossbred semen. In an initial series of animals, pregnant gilts were slaughtered on Days 9.5, 11, 11.75, 12, 13.5, 15.5, 15.75 and 15.75 of pregnancy, respectively (Day 0 defined as onset of standing heat). For each of these gilts, jugular blood samples were taken every 4h from the eighteenth day of the previous estrous cycle until the fifth day after onset of estrus. Plasma luteinizing hormone (LH) concentrations were quantified by radioimmunoassay as described by De Rensis et al. (1993). Based on the time of the preovulatory LH surge and the predicted time of ovulation, a revised estimate of day of pregnancy was made (Fig. 3.1 and Table 3.1).

Since a limited amount of RNA samples could be extracted from blastocysts at these early developmental stages, a secondary series of animals were slaughtered on Days 10.5, 11, 12, 14, 16 of pregnancy (Day 0 defined as onset of standing heat), respectively,

to obtain sufficient blastocyst materials for completing the analysis of gene expression in this experiment. In addition, the uterine fluids of pregnant gilts obtained from Day 3, 5.5, 7, 9, 10.5, 12 and 14 of pregnancy (Day 0 defined as onset of standing heat) were used for examination of proMMP-2, proMMP-9 and TIMPs levels.

B. Collection of Cells, Blastocysts and Endometrial tissues

The reproductive tract was recovered within 10 minutes of slaughter. Uterine horns were excised and individually flushed with physiological saline. Blastocysts were collected from the uterine flushings, pooled within individual pigs, and rinsed briefly in physiological saline to remove uterine debris prior to snap-freezing in liquid nitrogen.

Sections of endometrial tissues were harvested from the mid-section of each uterine horn of the initial series of pregnant gilts. The endometrial tissues were immediately snap-frozen in liquid nitrogen after collection. All samples were stored at – 80 °C.

A porcine trophoblast cell line, Jag-1, was included for comparison of gene expression, and the cultured trophoblast cells were harvested before they reached confluence, as described previously (Ramsoondar et al., 1994).

C. RNA Extraction

Total cellular RNA was extracted using the TRIzol reagent according to the manufacturer's instructions (Gibco/BRL, Gaithersburg, MD, USA). TRIzol reagent was directly added to the frozen embryonic tissues or cultured cells, whereas frozen endometrial tissues were first ground to a powder in a mortar and pestle in liquid nitrogen prior to addition of TRIzol reagent. RNA samples were dissolved in sterile distilled water and quantified by UV-spectrophotometer.

D. Primers and RT-PCR

All PCR primer sequences and the expected product sizes are listed in Table 3.2. Design of PCR primers for MMP-1, MMP-2, MMP-8, MMP-11, MMP-13, MMP-14, TIMP-1 and TIMP2 were based on the conserved region of human and mouse cDNA sequences. MMP-9, TIMP3 and uPA primers were synthesized according to Menino et al. (1997), and primers for G3PDH and β-actin were designed according to Yelich et al. (1997).

Reverse transcription (RT) was carried out with Expand™ reverse transcriptase (Boehringer Mannheim) according to the manufacturer's instructions. Briefly, cDNA was synthesized at 42°C for 1 hr from 1 μg of total RNA with 50 U of Expand™ reverse transcriptase in a total reaction volume of 20 μl, containing 5 μM oligo$(dT)_{15}$, 1 mM each of dATP, dCTP, dGTP and dTTP, 50 mM Tris-HCl pH 8.3, 40 mM KCl, 5 mM $MgCl_2$, and 0.5% Tween 20 (v/v). The RT reaction could be scaled up as needed and the final reaction mixture was stored at 4°C prior to use. Aliquots from the same RT reaction were used to amplify each of the target genes including G3PDH (Table 3.3) for each RNA sample, and one reaction mixture was prepared for PCR amplification of all target genes in that sample. Therefore, the PCR amplification of all target genes for each sample was carried out under the same conditions, except that the amount of cDNA templates, the annealing temperature and the cycle number of PCR were optimized empirically to each of these target genes (Table 3.2). All PCR reactions were carried out in a Perkin Elmer GenAmp 9600 Thermocycler in a reaction volume of 50 μl, containing 1.25U *Taq* polymerase (Gibco/BRL), 0.5 μM of each specific primer, 20 mM Tris-HCl pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$ and 0.2 mM dNTP mixture. The identities of the amplified

products were confirmed by sequencing and/or diagnostic restriction enzyme analysis. RT-PCR amplification was repeated on total RNA from each sample at least twice. Direct PCR amplification of total RNA without prior reverse transcription did not yield products with the predicted sizes for any of the target genes.

E. Substrate Gel Electrophoresis (Zymography and Reverse Zymography)

Uterine flushings were collected and centrifuged at 1,500 xg for 10 min at 4°C to remove tissue debris. Total protein was quantified using the BCA protein assay kit (Sigma, St. Louis, MO, USA) according to the manufacturer's instructions. Zymography was carried out as described by Lantz and Ciborowski (1994), and reverse zymography was performed as described by Staskus et al. (1991). Briefly, samples balanced for protein content were mixed with SDS loading buffer, and loaded on 15 % (w/v) SDS-polyacrylamide gel containing 1 mg/ml gelatin (Type A) (Sigma, Mississauga, ON, Canada). After separation by electrophoresis at 4 °C, enzymes were renatured by incubating the gel once in 2.5% (w/v) Triton X-100 (Sigma, St. Louis, MO, USA) for 30 min. The gel was then washed twice in 50 mM Tris-HCl pH 7.4 for 15 min, which was followed by incubation for 26 hr at 37 °C in gelatinolysis buffer (50 mM Tris-HCl pH 7.4 containing 10 mM $CaCl_2$). The gel was stained with Amido Black. Degradation of gelatin in the gel due to the action of proteinases was revealed as transparent bands. For reverse zymography, a crude mixture of gelatinases from serum-free conditioned medium of HT1080 cells (Giambernardi et al., 1998) was incorporated into 20% (w/v) polyacrylamide gel containing gelatin. Electrophoresis was carried out exactly as described above for zymography. Inhibitor activities were revealed as dark bands against the partially clear background.

RESULTS

A. Assessment of Developmental Stages of Conceptuses

Most of the studies of reproduction in pigs have designated onset of estrus (standing heat) as Day 0 of pregnancy. Recent data have confirmed that onset of estrus is not an accurate predictor of the time of ovulation since ovulation can occur from 10 to 85 h after onset of estrus in sows and from 23 to 48 h in gilts (Soede and Kemp, 1997). The average duration between the onset of estrus and ovulation is 44 hr with a range between 30 to 60 h based on an ultrasound study of 90 gilts used in our laboratory (Alemeida et al., 1999 manuscript under review). Therefore, the developmental stages of conceptuses from individual gilts used in our laboratory could vary by as much as 30 h if they are estimated by onset of estrus.

In order to assess developmental stages of conceptuses more accurately, the peak of the preovulatory LH surge was estimated for the initial series of gilts in the present study by measuring the LH concentration in the jugular blood during the peri-ovulation period. It is known that ovulation occurs approximately 30-35 h after the peak of the preovulatory LH surge (Kemp and Soede, 1997; Soede et al., 1994). Therefore, Day 0 of pregnancy is defined in the present study as the period between 14 hr before and 10 hr after the peak of LH surge (Fig. 3.3). In this way, the variation in the developmental stage estimated for conceptuses from individual gilts could be theoretically reduced from 30 h to 4.5 h (2 h from the estimation of the peak of LH surge plus 2.5 h from the interval between the ovulation and the peak of LH surge). As a result, the developmental stages of conceptuses could be defined more precisely. Despite this, the developmental stages of

conceptuses from the initial series of gilts in the present study followed the same temporal order whether they were determined by onset of estrus or by the peak of the LH surge, with the exception of the two gilts No. 170 and 367 (Table 3.1). Since the LH data for the secondary series of gilts in the present study were not available, the developmental stages for the conceptuses from these gilts were based on the onset of estrus. Where possible, the data is presented in terms of both methods of estimation.

B. Expression of MMP-2 and MMP-9

Transcripts of G3PDH were expressed at relatively constant levels in both endometrial tissues and blastocysts (Fig. 3.2F, 3.3F & 3.3N). Expression of MMP-2 (gelatinase A) transcripts was variable in the endometrium (Fig. 3.2G), whereas MMP-2 transcripts increased gradually in blastocysts obtained between Days 11 and 15 of pregnancy (Fig. 3.3G). ProMMP-2 was detected by zymography in uterine flushings of gilts obtained between Day 3 and 14 of pregnancy, with elevated levels on Day 3 and Day 14, respectively (Fig. 3.4A&3.4C). MMP-9 (gelatinase B) transcripts were not detectable in the endometrium unless a higher amount of cDNA template (10 µl) and a higher number of PCR cycles (45) was used for amplification (Fig. 3.2C). Under these conditions, transcripts of MMP-9 were still not detectable in any blastocysts examined, although they were revealed at low levels in Jag-1 cells (Fig. 3.3C). ProMMP-9 was only detected in uterine flushings obtained on Day 3 and 7 of pregnancy, respectively, and not on Days 9, 10.5, 12, 14 of pregnancy (Fig. 3.4A).

C. Expression of MMP-14, uPA and TIMPs

MMP-14 transcripts were constantly detected at high levels in endometrial tissues obtained between Day 8.7 and 15 of pregnancy (Fig. 3.2K). MMP-14 transcripts

were observed at low levels in blastocysts of Day 11 but increased dramatically in blastocysts obtained between Day 12 and 15 (Fig 3.3K). Expression of uPA transcripts slightly decreased in endometrial tissues collected between Day 9 and 15.75 of pregnancy (Fig. 3.2M), whereas it exhibited a biphasic pattern in blastocysts obtained between Day 11 and 15, with peak levels on Day 11.2 and Day 15, respectively (Fig. 3.3M). In endometrial tissues obtained between Day 9 and 15.75 of pregnancy, TIMP-1 and -3 transcripts were detected at variable levels, while TIMP-2 transcripts increased slightly (Fig. 3.2D, 3.2E & 3.2L). Transcripts of TIMP-2 were not detected in blastocysts obtained on Day 11 (gilt No. 399), but increased in blastocysts obtained between Day 11 (gilt No. 153) and Day 15 of pregnancy (Fig. 3.3L). Levels of TIMP-1 and TIMP-3 transcripts were lower in blastocysts obtained on Day 11, and dramatically increased in blastocysts collected on Day 12, 14 and 16 (Fig. 3.3D & 3.3E). TIMP-2 activity was not detected by reverse zymography in uterine flushings of pregnant gilts collected on Day 3, 5.5 and 7 of pregnancy, but was observed in uterine flushings obtained between Days 9 to 14 of pregnancy, with higher levels on Day 14 (Fig. 3.4B&3.4C).

D. Expression of Other MMP Transcripts

Expression of three types of interstitial collagenases, MMP-1 (collagenase-1), MMP-8 (collagenase-2) and MMP-13 (collagenase-3) was observed during the peri-implantation period. MMP-1 transcripts were only detected at low levels in endometrial tissues collected on Day 12.2 and 15 of pregnancy (Fig. 3.2A). Expression of MMP-1 transcripts was not detected in blastocysts obtained on Day 10.5, 11 and 12, although it was observed at low levels in blastocysts obtained on Day 14 and 16 (Fig. 3.3A). In endometrial tissues, expression of MMP-8 transcripts was not constant and was high on

Day 8.7, 11 (gilt No. 399), 11.2 and 12.2 of pregnancy and low on Day 14.5 of pregnancy (Fig. 3.2B). In contrast, expression of MMP-8 transcripts was up regulated gradually in blastocysts obtained between Days 10.5 and 16 of pregnancy (Fig. 3.3B). MMP-13 transcripts were not detected in any endometrial tissues (Fig. 3.2J). However, MMP-13 transcripts were detected at low levels in blastocysts obtained on Day 14.5 of pregnancy but not in blastocysts obtained from other developmental stages (Fig. 3.3J).

MMP-11 transcripts were observed at low levels in endometrial tissues obtained on Day 14, 14.5 and 15 of pregnancy but not in endometrial tissues collected from other developmental stages (Fig. 3.2I). However, MMP-11 transcripts were not detected in blastocysts from all developmental stages examined (Fig. 3.3I). Expression of MMP-7 (matrilysin) transcripts was not detected in any endometrial tissues examined (Fig. 3.2H). In contrast, MMP-7 transcripts were detected in blastocysts obtained on Day 15 but not in blastocysts collected at other developmental stages (Fig. 3.3H).

DISCUSSION

Convincing evidence demonstrates that the invasive properties of mouse and human trophoblast cells are due to the secretion of matrix metalloproteinases (MMP), especially MMP-9, that can degrade ECM components (Morgan et al., 1998; Bass et al., 1997; Alexander et al., 1996; Hurskainen et al., 1996). In the mouse, it has been shown that MMP-9 is the predominant metalloproteinase expressed by trophoblast cells of the implanting embryo and TIMP-3 is the major metalloproteinase inhibitor in the maternal decidua. During mouse embryo implantation, MMP-2, TIMP-1 and TIMP-2 were expressed mainly in the undifferentiated stroma outside the decidua, and TIMP-3 was

expressed in primary and mature decidual cells during their differentiation (Alexander et al., 1996). Expression of MMP-9 was not detected in uterine stromal cells except of those at the site of implantation. It has also been shown that MMP-9 activity is a rate-limiting factor for mouse trophoblast cells to invade and degrade ECM *in vitro* (Behrendtsen et al. 1992). Therefore, pronounced expression of MMP-9 in trophoblast giant cells, coupled with high-level expression of TIMP-3 in the stroma surrounding the embryo, contributes to the regulated invasive implantation in the murine uterus (Das et al., 1997; Leco et al., 1996; Reponen et al., 1995; Harvey et al., 1995). Similarly, expression of both MMP-9 and TIMP-3 is up-regulated in human cytotrophoblasts along the invasive pathway, which plays an important role in regulating the depth of blastocyst invasion in the human uterus (Bass et al., 1997; Shimonovitz et al., 1994; McMaster et al., 1994; Librach et al., 1991). Furthermore, MMP-9 has been shown to be the crucial factor for human trophoblast cells to invade ECM *in vitro* (Morgan et al., 1998). Therefore, MMP-9 appears to be a common effector for trophoblast invasion during embryo implantation in mice and humans (Salamonsen, 1999).

During normal embryo implantation in the pig, trophoblast cells of blastocysts do not penetrate the basement membrane of the endometrium. In the present study, MMP-9 transcripts were not detected in blastocysts although they were weakly expressed in the endometrium during the peri-implantation period and in trophoblast cells cultured *in vitro*. This result is in agreement with a previous study in which MMP-9 transcripts were not detected in porcine blastocysts and the endometrium at the time of embryo-uterine contact by RT-PCR and *in situ* hybridization, although they could be detected by Southern blot analysis of RT-PCR products (Menino et al., 1997). These earlier results

were extended in the present study by zymography data showing that MMP-9 activity was not detected in uterine fluids representing Day 9 to 14 of pregnancy. In contrast, expression of TIMP-1, TIMP-2 and TIMP–3 transcripts was observed in both blastocysts and the endometrium, and TIMP-2 activity was also detected in uterine fluids from gilts representing Day 9 to 14 of pregnancy. Taken together, these results suggest that little or no production of MMP-9, coupled with high levels of TIMP-1, TIMP-2 and TIMP–3 expression in blastocysts during embryo attachment, could be responsible for the non-invasive type of implantation seen in pigs. These results are in agreement with previous studies on the role of MMP-9 in regulating embryo implantation in mice and humans with invasive hemochorial placentation.

MMP-9 transcripts and active MMP-9 enzyme were not detected in blastocysts and uterine fluids, respectively, at the time of embryo-uterus contact by the very sensitive approaches used in the present study. Therefore, the trophoblast cells of implanting porcine blastocysts do not appear to produce significant levels of MMP-9 during normal pregnancy. In contrast, low levels of MMP-9 transcripts were observed in Jag-1 trophoblast cells in the present study, and significant levels of proMMP-9 activity was also observed in conditioned media from Jag-1 cells (data not shown). In addition, the Jag-1 cell line was able to invade matrigel (artificial basement membrane) *in vitro* in our laboratory (Chai et al., unpublished results). These data suggest that porcine trophoblast cells are invasive *in vitro*, which provides further evidence at the molecular level for previous histological observations in which porcine trophoblast cells appear to be invasive after porcine conceptuses are transferred to ectopic sites (Samuel, 1971and 1972; Samuel and Perry, 1972). The differential behaviors of porcine trophoblast cells

under *in vivo* and *in vitro* conditions suggest that there might be some negative regulatory mechanisms, within the blastocsyt or in the uterine fluid, limiting the production of MMP-9 in trophoblast cells *in vivo*, possibly at the transcriptional level.

In addition to embryo implantation, tissue remodeling involving the actions of MMPs is a component of several other processes occurring during mammalian embryogenesis, including cell migration, cell to cell interactions, and embryo expansion (Brenner et al., 1989). It has been shown that metalloproteinases have distinct roles in the differentiation and migration of murine parietal endoderm, the first embryonic migratory cells that adhere to ECM secreted from trophoblast cells (Behrendtsen and Werb, 1997). MMP-2 is secreted as a proenzyme that can form a specific stoichiometric complex with TIMP-2. Activation of the secreted proenzymes and interaction with TIMP-2 determine the net enzymatic activity in the extracellular space. The cell-surface activation of proMMP-2, involving the formation of a tri-molecular complex of MMP-14.MMP-2.TIMP2, is considered to be critical for cell migration and invasion. Activated MMP-14 was shown to act as a cell surface receptor for TIMP-2, and this bi-molecular complex in turn acts as a receptor for MMP-2 to recruit proMMP-2 to the cell surface (Strongin et al., 1995). Moreover, TIMP-2 bound to the plasma membrane was shown to be the specific inhibitor of the cell surface-activated MMP-2 (Itoh et al., 1998; Strongin et al., 1993). Activation of MMP-2 on the cell surface provides a basic mechanism for spatially regulated extracellular proteolysis. More interestingly, a number of investigators have recently shown that MMP-2 is co-expressed temporally and spatially with its activator MMP-14 and its inhibitor TIMP-2 in mouse tissues during embryogenesis, and in human fetal membranes (Apte et al., 1997; Kinoh et al., 1996; Fortunato et al., 1998). In the

present study, expression of MMP-2, MMP-14 and TIMP-2 transcripts was up-regulated in embryonic tissues with the progression of embryonic development, and elevated levels of proMMP-2 and TIMP-2 were also observed in uterine fluids of pregnant gilts at the time of embryo-uterus contact. The co-expression of MMP-2, TIMP-2 and MMP-14 in porcine blastocysts during the peri-implantation period might contribute to the spatially regulated extracellular proteolysis required for the extraembryonic mesoderm migration from the primitive streak region in porcine blastocysts.

The levels of proMMP-2 were shown to be very high in uterine fluids of pregnant gilts obtained between Day 9 to 14 of pregnancy in the present study. Since MMP-2 transcripts were both expressed in endometrial tissues and blastocysts obtained between Day 11 to 15 of pregnancy, the high levels of proMMP-2 in the uterine fluid could be contributed by secretion from both implanting blastocysts and the endometrium. This enhanced secretory activity might be a response to the increased embryonic estrogen content known to exist in uterine fluids during this period (Gadsby et al., 1980; Geisert et al., 1982b; Fischer et al., 1985). Additional protein species with strong gelatinolytic activity were also detected by zymography of uterine flushings from a pregnant gilt obtained on Day 14 of pregnancy. These protein species might represent the active form (62 kDa) and low molecular mass forms of MMP-2, or proMMP-3, both of which also degrade gelatin (Salamonsen et al., 1995; Nagase, 1991).

Although expression of TIMP-1, TIMP-2 and TIMP-3 transcripts was detected in both blastocysts and the endometrium, strong activity of TIMP-2, but not TIMP-1 and TIMP–3, was observed in uterine fluids in the present study. These results are consistent with a previous study in which transcripts of TIMP-1 and TIMP-3 were mainly localized

in endodermal cells, whereas those of TIMP-2 were detected in all cells of the blastocyst (Menino et al., 1997). In addition, TIMP-3 is found exclusively in the ECM of a large number of cultured human cells but not in the conditioned media (Kishnani et al., 1995). High levels of TIMP-2 in the uterine fluid of pregnant gilts could play a major role in preventing potential damage to the integrity of the uterine epithelium by the large amount of proMMP-2, existing in the uterine lumen as shown in the present study, at the time of embryo-uterus contact. However, levels of TIMP-1, -2 and –3 transcripts were shown to be up-regulated during the peri-implantation period in the present study, which suggests that TIMP-1, -2 and –3 might all be involved in maintaining the balance between ECM deposition and degradation in blastocysts during the mesoderm outgrowth.

It is interesting to note that the expression of uPA transcripts appears to be biphasic in porcine blastocysts during the peri-implantation period examined. This extends a previous observation on uPA enzyme activities released by porcine blastocysts isolated from pregnant pigs representing the same stages of pregnancy (Fazleabas et al., 1983). Coincidentally, expression of uPA observed in the present study is temporally correlated with estrogen synthesis in porcine blastocysts that has been previously shown to peak around Day 11 and Day 16 of pregnancy, respectively (Pusateri et al., 1990, Geisert et al., 1987; Heap et al., 1981; Zavy et al., 1980). It is known that estrogen could down-regulate the expression of uPA mRNA in a dose-dependent fashion in human breast cancer cells (Leverson et al., 1998). Therefore, we hypothesize that embryonic estrogen might be responsible for dramatic changes in the production of uPA by blastocysts. Together with earlier *in situ* hybridization data showing that uPA transcripts were mainly localized to the extraembryonic endoderm with little expression in the

trophoblast cells (Menino et al., 1997), our results suggest that the proteolytic cascade driven by uPA through plasmin might be required for early porcine embryogenesis. The ultimate activity of uPA in the uterine lumen released from blastocysts could be regulated by inhibitors of both uPA and plasmin, existing in the uterine lumen as shown by Mullins et al. (1980) and Fazleabas et al. (1983).

Expression of MMP-1, MMP-8 and MMP-13 transcripts was observed in porcine blastocysts in the present study, which suggests that they might be involved in the tissue remodeling occurring during porcine embryogenesis. Low levels of MMP-1 transcripts were detected in endometrial tissues of pregnant gilts obtained at the time of embryo-uterine contact in the present study, which is consistent with a previous observation in uterine tissues of pregnant ewes (Hampton et al., 1995). MMP-8 (collagenase-2) is also known as neutrophil collagenase because of its exclusive expression in the neutrophil under inflammatory conditions (Jeffrey, 1998). The present study showed that MMP-8 transcripts were expressed at variable levels in the endometrium, suggesting that neutrophils might be recruited to the endometrium in response to the implanting blastocysts. However, expression of MMP-8 transcripts is found to increase gradually in porcine blastocysts during the peri-implantation in the present study, which correlates with the formation of extraembryonic mesoderm. These results are in agreement with the observation of MMP-8 expression at late stages during mouse embryogenesis, coinciding with the appearance of hematopoietic cells, and also in the *post partum* uterus (Balbin et al., 1998). MMP-13 transcripts were not detected in the endometrium of pregnant pigs in the present study, which extends a previous observation in the mouse uterus (Das et al., 1997). MMP-7 was first discovered in the involuting rat

uterus and has been termed the uterine metalloproteinase. It is capable of degrading a wide range of ECM components and can activate several other MMPs including collagenases (Woessner, 1996; Wolf et al., 1996). However, MMP-7 transcripts were not observed in porcine endometrial tissues during the stages of pregnancy examined, were observed in Jag-1 cells, and were strongly expressed in blastocysts at the time of embryo-uterine contact in the present study. These data suggest that MMP-7 might be involved in the activation of proMMP-2 in porcine blastocysts as suggested in the activation of human proMMP-2 by Crabbe et al. (1994).

Previous studies on gene expression during porcine embryo development have been carried out with reference to onset of standing heat. Recently, Soede and Kemp (1997) reported that the onset of standing heat was not a good predictor of the time of ovulation. Therefore, particularly in early gestation, determination of developmental stages of porcine embryos based on the onset of standing heat might not be very accurate. Based on known endocrine relationships, the preovulatory LH surge can be used as a more accurate predictor of the time of ovulation than the onset of standing heat, and therefore of true developmental age. As shown in the present study (Table 3.1), developmental stages of embryos isolated from pregnant gilts No. 399 and No. 153 were Days 11 and 11.75, respectively, based on the onset of standing heat, but they were both Day 11 according to the time of preovulatory LH surge. In contrast, developmental stages of embryos isolated from pregnant gilts No. 170 and No. 367 were both Day 15.75 based on the onset of standing heat, but they were Days 14.5 and 15.0, respectively, according to the time of preovulatory LH surge. In future studies that examine the gene expression during early embryo development, we believe that accurate assessment of the

developmental stages of embryos and/or conceptuses is needed. In addition, it should be noted that the number of animals used in the present study is limited, although the number of blastocysts is reasonably sufficient for representing individual developmental stages of embryos.

In summary, we have examined the temporal expression of MMPs and their regulators in the endometrium and blastocysts during the peri-implantation period. Expression of these protein factors correlate with conceptus implantation and early embryogenesis in the pig, which are the two major biological processes coordinated during this period. Little or no expression of MMP-9 in blastocysts during the peri-implantation period may be the basis for the non-invasive implantation/ attachment process seen in the pig. The co-expression of MMP-2, TIMP-2 and MMP-14 might contribute to the mesoderm migration during early embryogenesis in the pig.

Table 3.1 Determination of developmental stages of pregnant gilts based on the onset of estrus and the peak of the preovulatory LH surge, respectively

Pig No.	Day of pregnancy (based on onset of estrus)	Date and time of the peak of the LH surge	Date and time of slaughter	Interval between LH peak and slaughter (h)	Day of pregnancy (based the LH data)
179	9.50	May 6; 8:00	May 15; 10:00	16+8x24+10=218	(218-10)/24=8.7
399	11.00	Aug 18; 24:00	Aug 30; 10:00	11x24+10=274	(274-10)/24=11.0
153	11.75	April 26; 24:00	May 8; 10:00	11x24+10=274	(274-10)/24=11.0
354	12.00	Aug 18; 20:00	Aug 30; 10:00	4+11x24+10=278	(278-10)/24=11.2
172	13.50	May 2; 20:00	May 15; 10:00	4+12x24+10=302	(302-10)/24=12.2
387	15.50	Aug 20; 24:00	Sept 4; 10:00	14x24+10=346	(346-10)/24=14.0
170	15.75	April 29; 24:00	May 15; 10:00	15x24+10=370	(370-10)/24=15.0
367	15.75	Aug 20; 12:00	Sept 4; 10:00	12+14x24+10=358	(358-10)/24=14.5

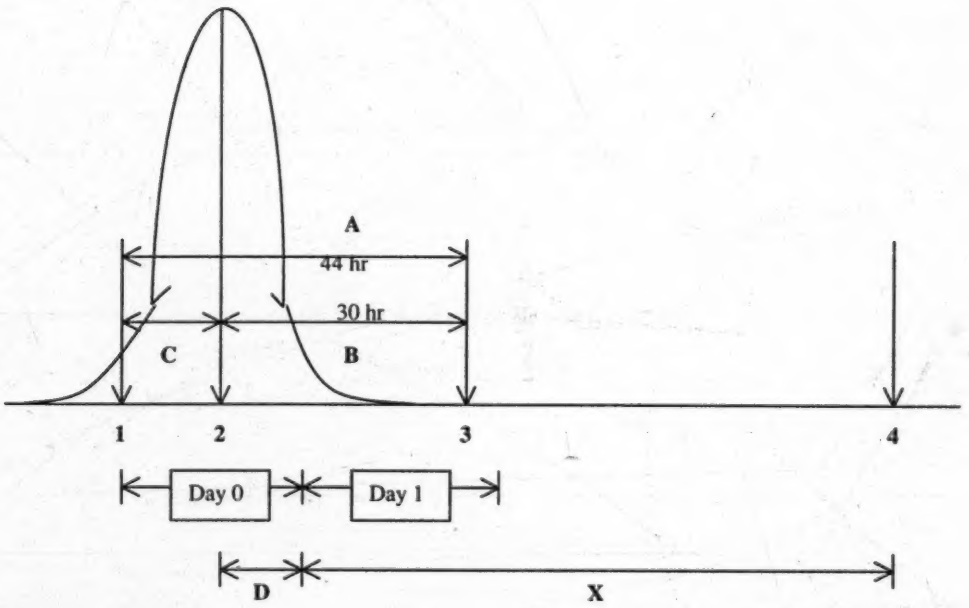
Table 3.2 PCR primers for target genes and their sequences

Target Genes	Primer sequences#	Predicted size (bp)	Source*
MMP-1	GCAGCCCAGATGTGGGGTGCCCG ACACTTCTGGGGTTTGGGGGCCG	571	
MMP-2	CTTCCCCCGCCAGCCCAAGTGGG GGTGAACAGGGCTTCATGGGGGC	510	
MMP-7	CCCAAAGAATGGCCAAGTTC TGCAGAAGCCCAGATGTGGA	420	
MMP-8	GATGGACCCAATGGAATCCTTGC TCATAGCCACTCAGAGCCCA	550	
MMP-9	ACGTGGACATCTTCGACGC CGAACCTCCAGAAGCTCTGC	357	Menino et al., 1997
MMP-10	GCTCTTCAGTGTGTGTGTCACC GGAAATGAAGTCCAAGCAGG	323	
MMP-11	GCTCTTCTTCTTGGCAGGC AACGCCAATAGTCTCCACCTCG	313	
MMP-13	TCTGGTCTGCTGGCTCACGC TAGGCAGCATCAATACGGTTGG	472	
MMP-14 (MT1-MMP)	ACCATGAAGGCTATGAGGCGCCC GTTGATGGATGCAGGCAGGCCCC	860	
uPA	GTCTGGTGAATCGAACTGTGGC GGCTGCAAACCAAGGCTG	538	Menino et al., 1997
TIMP-1	GTACCTGCGTCCCACCCCACC GGCAGGCAGGCCAGGTGGCGG	504	
TIMP-2	CCTCCTGCTGCTGGGGACGCTGC AGTCCTGGTGGCCTGCTTACGGG	643	
TIMP-3	GACCCCTTGGCTCGGGCTCATCG GCTGGTCCCACCTCTCCACGAAG	379	
G3PDH	ACCACAGTCCATGCCATCAC TCCACCACCCTGTTGCTGTA	452	Yelich et al., 1997
β-Actin	ATCTTGATCTTCATGGTGCTGGGC ACCACTGGCATTGTCATGGACTCT	545	Yelich et al., 1997

#All primer sequences were listed from 5' end to 3' end. * Primers were designed according to conserved regions of human and mouse cDNA sequences obtained from GenBank database unless indicated.

Table 3.3 PCR cycling conditions for amplification of target genes

Target genes	Annealing temp (°C)	No. of cycles	cDNA (µl)
G3PDH & β-Actin (Co-amplification)	60	35	1
G3PDH (Alone)	57	35	0.4
MMP-1	57	35	5
MMP-2	65	35	4
MMP-7	57	35	4
MMP-8	57	45	4
MMP-9	57	45	10
MMP-10	57	35	4
MMP-11	57	35	4
MMP-13	57	35	4
MMM-14 (Mt1-MMP)	57	35	4
uPA	57	35	5
TIMP-1	57	35	5
TIMP-2	65	35	4
TIMP-3	57	35	5



Point 1: Onset of estrus.
Point 2: The peak of LH surge.
Point 3: Ovulation.
Point 4: Slaughter
A: the average duration (44 hr) between the ovulation and observed onset of estrous behavior from 90 gilts.
B: the duration between points 2 and 3 (based on previous endocrine studies).
C: =A – B = 44 – 30 =14 hr
D: = 24 – 14 = 10 hr
X: Total days of pregnancy

Formula: X = (Interval duration between points 2 and 4 — D) / 24.

Figure 3.1 Diagram showing different points of time at which predicted onset of estrus and ovulation occur in relation to the peak of the LH surge.

Figure 3.2 RT-PCR amplification of MMP-1 (A), MMP-2 (G), MMP-7 (H), MMP-8 (B), MMP-9 (C), MMP-11 (I), MMP-13 (J), MMP-14 (K), uPA (M), TIMP-1 (D), TIMP-2 (L), TIMP-3 (E) and G3PDH (F) transcripts in the endometrium during the peri-implantation period. The same series of pregnant gilts were used for examination of all target genes, and their developmental stages were indicated above the gels. Days of pregnancy in top panel were determined based on the peak of the LH surge, and those in the bottom panel were determined relative to the onset of estrus. N, negative control. PCR was carried out with 35 cycles for each target gene in this experiment, except for amplification of MMP-8 and MMP-9 where 45 cycles applied. RT-PCR products were analyzed in 1.2 % agarose gel stained with ethidium bromide. The sizes of PCR products for target genes were indicated on the left side of gels. G3PDH was amplified as an internal control for gene expression.

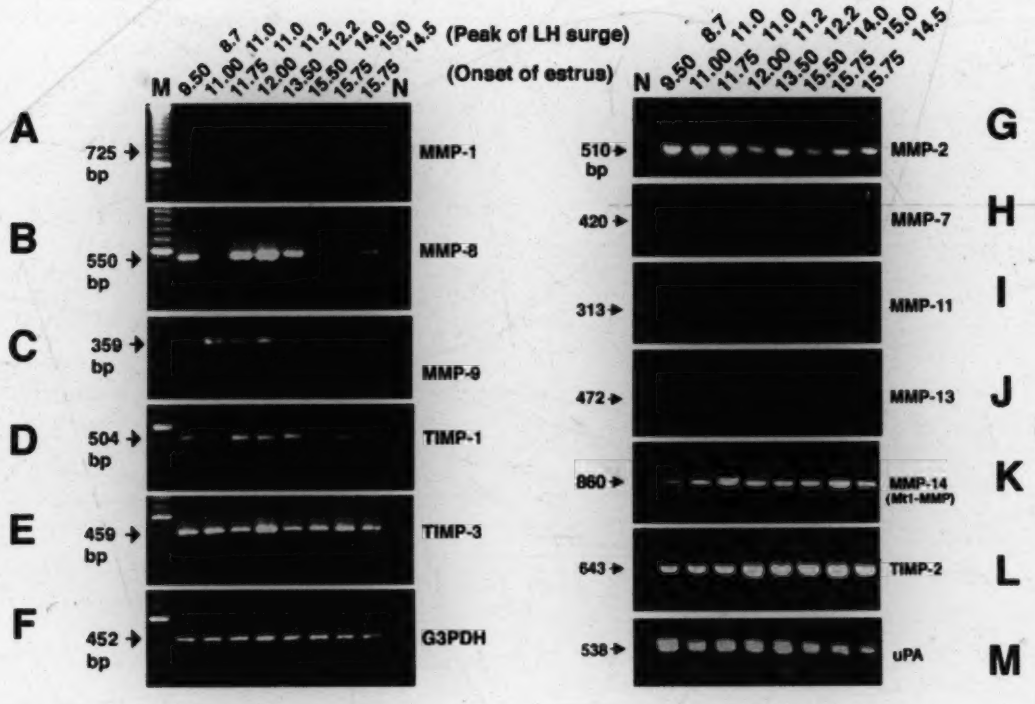

M
9.50
8.7
11.00
11.0
11.75
11.0
12.00
11.2
13.50
12.2
15.50
14.0
15.75
15.0
15.75
14.5
N
(Peak of LH surge)
(Onset of estrus)
A
725 bp
MMP-1
B
550 bp
MMP-8
C
359 bp
MMP-9
D
504 bp
TIMP-1
E
459 bp
TIMP-3
F
452 bp
G3PDH
G
510 bp
MMP-2
H
420
MMP-7
I
313
MMP-11
J
472
MMP-13
K
860
MMP-14
(Mt1-MMP)
L
643
TIMP-2
M
538
uPA

Figure 3.3 RT-PCR amplification of MMP-1 (A), MMP-2 (G), MMP-7 (H), MMP-8 (B), MMP-9 (C), MMP-11 (I), MMP-13 (J), MMP-14 (K), uPA (M), TIMP-1 (D), TIMP-2 (L), TIMP-3 (E), G3PDH (F) and G3PDH & beta-actin (N) transcripts in blastocysts during the peri-implantation period. Two different series of pregnant gilts were used in this experiment, and their developmental stages were indicated above the gels. Days of pregnancy in top panel were determined based on the peak of the LH surge, and those in the bottom panel were determined relative to the onset of estrus. T, Jag-1 cells; N, negative control. PCR was carried out with 35 cycles for each target gene in this experiment, except for amplification of MMP-8 and MMP-9 where 45 cycles were applied.. RT-PCR products were analyzed in 1.2 % agarose gel stained with ethidium bromide. The sizes of PCR products for target genes were indicated on the left side of gels. Beta actin and/or G3PDH were amplified as internal controls for gene expression.

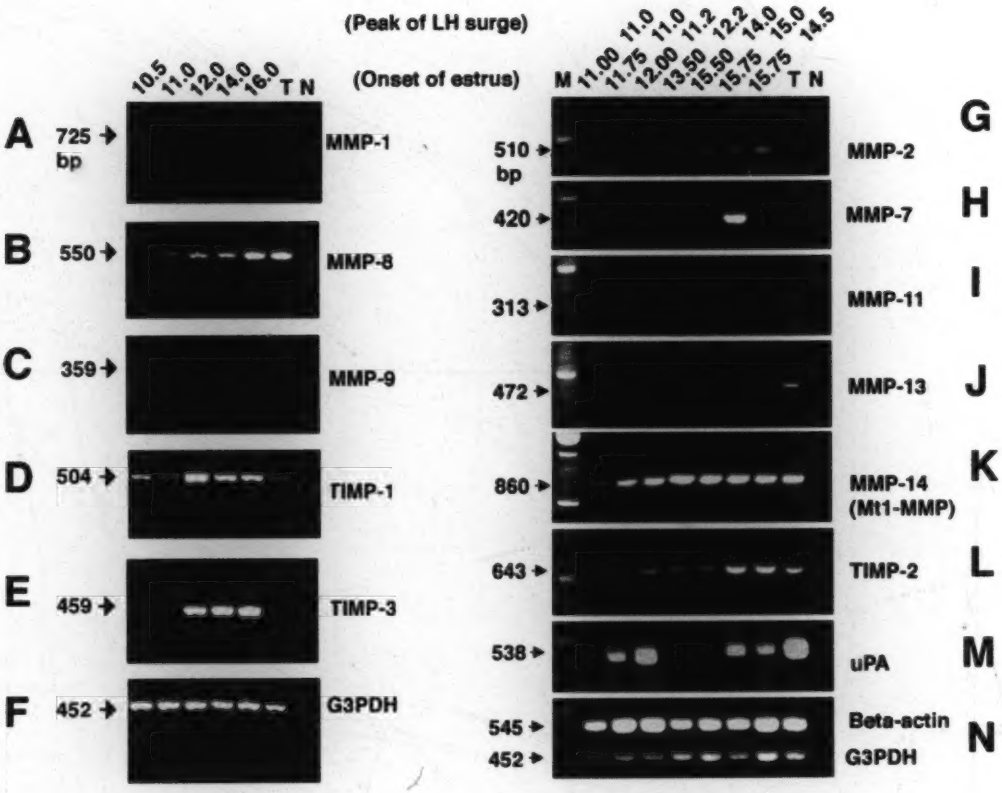
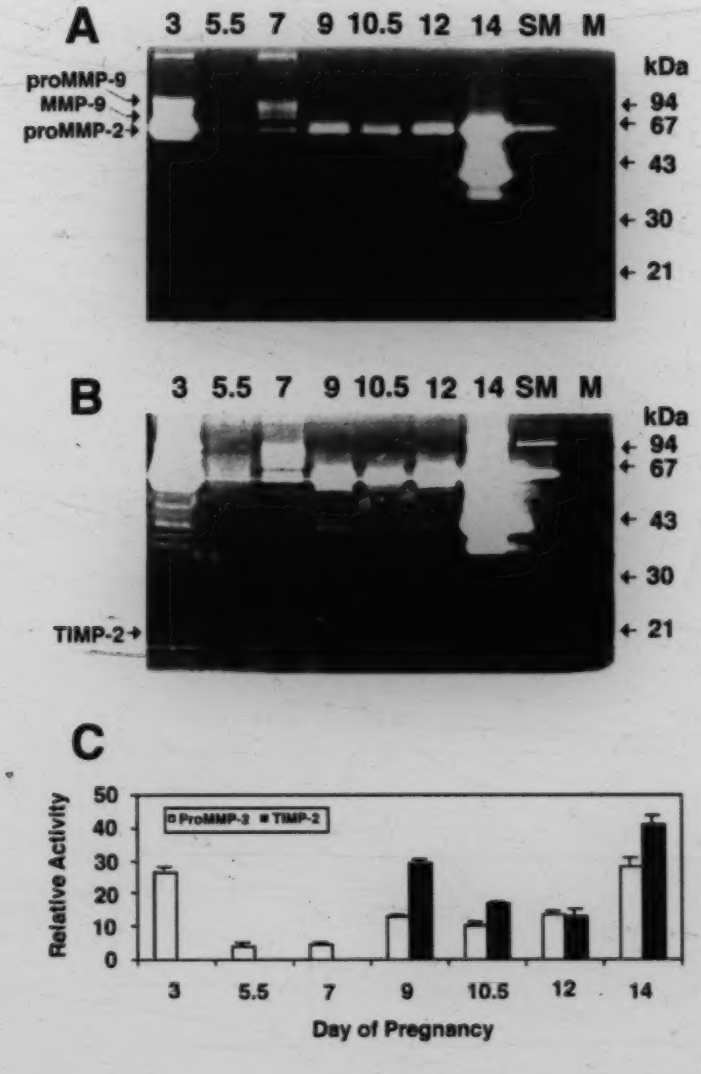

(Peak of LH surge)
(Onset of estrus)
10.5 11.0 12.0 14.0 16.0 T N
M 11.00 11.75 12.00 13.50 15.50 15.75 15.75 11.0 11.0 11.2 12.2 14.0 15.0 14.5 T N
A
725 bp
MMP-1
B
550
MMP-8
C
359
MMP-9
D
504
TIMP-1
E
459
TIMP-3
F
452
G3PDH
G
510 bp
MMP-2
H
420
MMP-7
I
313
MMP-11
J
472
MMP-13
K
860
MMP-14 (Mt1-MMP)
L
643
TIMP-2
M
538
uPA
N
545
Beta-actin
452
G3PDH

Figure 3.4 Gelatin zymographic (A) and reverse zymographic (B) analysis of MMPs and TIMPs in uterine flushings of gilts obtained on Days 3, 5.5, 7, 9, 10, 11 and 14 of pregnancy relative to onset of estrus, respectively. Total proteins of 10 μg were loaded on each lane. SM, total proteins of 20 μg from bovine skeletal muscle homogenate as positive controls for proMMP-2 and proMMP-9. M, molecular weight marker. (C) Relative levels of proMMP-2 and TIMP-2 in uterine fluids of pregnant pigs between Day 3 and 14 of pregnancy. Results were presented as the mean ± SEM of three separate experiments.


A
3 5.5 7 9 10.5 12 14 SM M
kDa
proMMP-9
MMP-9
proMMP-2
94
67
43
30
21
B
3 5.5 7 9 10.5 12 14 SM M
kDa
94
67
43
30
TIMP-2
21
C
ProMMP-2 TIMP-2
Relative Activity
50
40
30
20
10
0
3 5.5 7 9 10.5 12 14
Day of Pregnancy

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CHAPTER 4

EXPRESSION OF INTEGRIN TRANSCRIPTS IN BLASTOCYSTS DURING EARLY EMBRYOGENESIS, AND IN THE ENDOMETRIUM DURING EARLY GESTATION, IN THE PIG

INTRODUCTION

During pre-implantation embryonic development, a newly "hatched" porcine blastocyst consists of an inner cell mass (ICM) surrounded by a single layer of trophoblast, and has a spherical shape with a diameter of approximately 0.2 mm. It expands rapidly due to the inward transport of fluid by trophoblast cells. As the blastocyst expands, some of the ICM cells initiate an outgrowth along the inner surface of the trophoblast and subsequently form a complete layer of cells called the extraembryonic endoderm (Patten, 1948; Geisert et al., 1982a; Barends et al., 1989). Immediately after the endoderm has been established as a definite layer, the blastocyst undergoes a striking elongation from a spherical shape of about 8 to 10 mm in diameter to a filamentous shape of up to 100 cm in length (Geisert et al., 1982a). Along with the elongation process, the extraembryonic mesoderm initiates development from the primitive streak region and starts to migrate between the trophoblast and the extraembryonic endoderm (Patten, 1948). The trophoblast and the uterine epithelium become closely apposed to each other immediately after the blastocyst elongation is complete (Flood, 1991). This is followed by adhesion between these two cell layers around Day 14 of pregnancy, seen as the microvillous interdigitation between them by Day 16 of pregnancy (Day 0 designated as onset of estrus) (Dantzer, 1985; Keys and King, 1990). Implantation of the blastocyst is an essential step during mammalian pregnancy, leading to the formation of a placenta that supports embryonic/fetal development to term. Embryo implantation during pregnancy in

the pig is a non-invasive process resulting in simple attachment between the trophoblast and the uterine epithelium. This is in contrast to the invasive implantation seen in the mouse, where the trophoblast breaches the basement membrane of the uterine epithelium and penetrates into the underlying stromal tissue. It has been reported that one of the most important "windows" of embryonic mortality occurs from Day 13 to 20 of pregnancy in the pig, which is coincident with this embryo attachment process (Bazer, 1994). Therefore, elucidation of the mechanism(s) regulating the endodermal and mesodermal migration in blastocysts and the trophoblast-uterine epithelium attachment is essential for understanding aberrant processes of embryogenesis and/or implantation that may contribute to embryonic loss in this economically important domestic animal.

Early embryogenesis and attachment/implantation in the pig involves extensive cell-cell and cell-extracellular matrix interactions. These cellular interactions are mediated by several classes of adhesion molecules, one of the most important of which is the integrin family of cell surface adhesion receptors (Albelda and Buck, 1990; Hynes, 1992; Gille and Swerlick, 1996). Integrins function as transmembrane heterodimeric proteins consisting of α and β subunits linking cytoplasmic components of the cytoskeleton with the extracellular matrix (ECM) (Juliano, 1996; Yurochko, 1997). At least 24 integrin heterodimers can be formed from the 16 α subunits and 9 β subunits known, and nine of these have been detected in human uterine epithelium (Aplin, 1997). Integrins are modulated in the uterus during the reproductive cycle and early pregnancy in humans and rats (Nishida, Murakami and Otori, 1991; Lessey et al., 1992; Tabibzadeh, 1992; Lessey, 1997). Human and mouse embryos express specific integrins on their surface during pre-implantation development (Sutherland, Calarco and Damsky, 1993;

Campbell et al., 1995). The critical roles of integrins in differentiation, migration and invasion of trophoblast in both human and mouse have been reviewed by Damsky, Sutherland and Fisher (1993). Recently, Bowen and Hunt (1999) showed that integrins (α4, αv, β1 and β3) were also expressed within the developing murine placenta. As a first step in understanding the function of integrins during pregnancy in the pig, it was necessary to examine the expression and distribution of integrins in both the developing conceptus and in the female reproductive tract during pregnancy. Using indirect immunocytochemical techniques, the expression of selected integrin protein subunits was previously examined in porcine uterine epithelium and trophoblast *in vivo* and *in vitro* by Bowen, Bazer and Burghardt (1996 & 1997). Expression of integrin β1 subunit transcripts was previously examined in the early developing porcine conceptus by Yelich, Pomp and Geisert (1997a). Due to limitations on the amount of conceptus tissue during early embryogenesis, examination of the expression of gene transcripts in the whole embryo at this developmental stage has relied on the use of sensitive detection methods such as reverse-transcription polymerase chain reaction (RT-PCR) and *in situ* hybridization (Rappolee et al., 1989; Yelich, Pomp, D. & Geisert 1997a & 1997b). In the present study, the RT-PCR approach was used to characterize the expression patterns of transcripts for eight integrin subunits in blastocysts during early embryogenesis, in Day-28 placental tissue, in cultured trophoblast cells (Jag-1), and in the endometrium during early gestation in the pig. Our results support an important role for integrins in the processes of early embryogenesis, embryo implantation and placentation in the pig.

MATERIALS AND METHODS

A. Animals

Sexually mature gilts (PIC Camborough) of similar age and weight, and with two recorded estrous cycles, were heat-checked three times daily (08:00, 16:00 and 24:00) for onset of estrus (standing heat) with a vasectomized boar. At 16 and 24 hours after onset of estrus, gilts were artificially inseminated using fresh-pooled crossbred semen. All animals were handled in accordance with the Canadian Council on Animal Care (1993). Pregnant gilts were slaughtered as indicated below, and the reproductive tracts were recovered within 10 minutes of death.

B. Collection of Blastocysts, Endometrial and Placental Tissues

Uterine horns were excised at slaughter and individually flushed with physiological saline. Blastocysts were collected from the uterine flushings and rinsed briefly in physiological saline to remove uterine debris. Spherical blastocysts were classified according to their sizes into three groups: 1-2 mm (n=37), 5-6 mm (n=19) and 8-10mm (n=8) in diameter, which were collected from gilts on Days 9 (n=3), 10 (n=2) and 10.5 (n=1) of pregnancy, respectively (Table 4.1). Blastocysts representing elongated stages were found to clump together, and it was difficult to separate individual blastocysts from each other after they were flushed from the reproductive tract. Therefore, elongated blastocysts were pooled for individual gilts obtained on Days 11, 12, 14 and 16 of pregnancy (Table 4.1). The number of elongated blastocysts obtained from each pig was estimated by counting the free bulbous ends of aggregated blastocysts.

Sections of endometrial tissue were harvested from the mid-section of each uterine horn of gilts slaughtered on Days 9, 10, 10.5, 11, 12, 14, 16 and 28 of pregnancy

(Table 4.1). Additionally, endometrial samples of Days 3, 5.5, 7, 7.5, 11.75, 12.75, 13.5, 13.75, 15.5, 15.75 of pregnancy were collected previously in our laboratory. Sections of placental tissue were obtained from the allantochorionic membrane distal to the embryonic region from Day 28 conceptuses. All tissue samples were immediately snap-frozen in liquid nitrogen after collection and stored at –80 °C.

C. Cell Culture and Neutrophil Collection

A porcine trophoblast cell line, Jag-1, was included for comparison of gene expression, and Jag-1 cells were cultured and harvested as described previously by Ramsoondar et al. (1993).

Porcine blood was collected at slaughter in tubes containing an anticoagulant (heparin) and centrifuged at 200g for 10 min. The plasma was collected and diluted 1:2 with PBS buffer containing 2% (w/v) BSA. Diluted plasma (3-4 ml) was carefully layered onto a Histopaque (Sigma) (HP) step gradient with 3 ml HP-1077 over 3 ml HP-1119 in a 15-ml conical centrifuge tube (Boyum, 1968). Two distinct opaque layers could be observed following centrifugation at 700g for 30 min at room temperature. The lower layer containing neutrophils was recovered by careful aspiration and diluted with the buffer in a fresh tube. The mixture was centrifuged at 200g for 10 min, and the pellet was resuspended and washed twice with the buffer to remove excess HP reagent.

D. RNA Extraction

Total cellular RNA was extracted using TRIzol reagent according to the manufacturer's instructions (Gibco/BRL, Gaithersburg, MD, USA). TRIzol reagent was directly added to the frozen embryonic tissues, Jag-1 cells or neutrophils, whereas frozen endometrial tissues were first ground to a powder in a mortar and pestle in liquid nitrogen

prior to the addition of TRIzol reagent. RNA samples were dissolved in sterile distilled water and quantified by UV-spectrophotometer.

E. Primers and RT-PCR

PCR primers for integrin subunits used in the present study were designed according to conserved regions between human and mouse cDNA sequences, and primers for G3PDH were synthesized as described by Yelich, Pomp and Geisert (1997). PCR primer sequences and their expected product sizes are listed in Table 4.2.

Reverse transcription (RT) was carried out with Expand™ reverse transcriptase (Boehringer Mannheim) according to the manufacturer's instructions. Briefly, cDNA was synthesized at 42°C for 1 hr from 1 µg of total RNA with 50 U of Expand™ reverse transcriptase in a total reaction volume of 20 µl, containing 5 µM oligo$(dT)_{15}$, 1 mM each of dATP, dCTP, dGTP and dTTP, 50 mM Tris-HCl pH 8.3, 40 mM KCl, 5 mM $MgCl_2$, and 0.5% (v/v) Tween 20. Aliquots from the same RT reaction were used to amplify each of the target genes including G3PDH (Table 4.2) for each RNA sample. A master reaction mixture was prepared for PCR amplification of all target genes in that sample. Therefore, PCR amplification of all target genes for each sample was carried out under the same conditions, except that 0.4 µl of cDNA template were used for G3PDH while 5 µl of cDNA template were used for all integrin subunits. PCR was performed with an initial denaturation step at 94 °C for 2 min, followed by 35 cycles (denaturation at 94 °C for 30 seconds, annealing at 57 °C for 30 seconds and extension at 72 °C for 1 min), and a final extension step at 72 °C for 7 min. All PCR reactions were carried out in a Perkin Elmer GeneAmp 9600 Thermocycler in a reaction volume of 50 µl, containing 1.25U *Taq* polymerase (Gibco/BRL), 0.5 µM of each specific primer, 20 mM Tris-HCl pH 8.4,

50 mM KCl, 1.5 mM $MgCl_2$ and 0.2 mM dNTP mixture. The identities of amplified products were confirmed by sequencing and/or diagnostic restriction digestion analysis. RT-PCR amplification was repeated on total RNA from each sample at least twice. Direct PCR amplification of total RNA without prior reverse transcription did not yield products with the predicted sizes for any of the target genes (N lanes in Fig. 4.1 and Fig. 4.2).

RESULTS

A. Expression of Integrin Transcripts in Blastocysts

Transcripts of integrin subunits (α1, α2, α3, α4, α5, αv, β1 and β3) were examined in spherical and elongated blastocysts collected between Day 9 and 16 of pregnancy. In blastocysts at all stages examined, expression of G3PDH transcripts was both evident and relatively constant (Fig. 4.1A). Expression of α2, α3, αv, β1 and β3 transcripts exhibited similar overall patterns in blastocysts during the period examined (Fig. 4.1C-G). Expression was high in spherical blastocysts (1-2 mm in diameter; Lane S1), was dramatically down-regulated in spherical blastocysts (5-6 mm and 8-10 mm; Lanes S2 and S3), and progressively increased as blastocysts elongated between Days 11 and 16 (Lanes F1 to F4). It is interesting to note that transcripts of all integrin subunits examined were not detected in the 8-10 mm spherical blastocysts, even though levels of G3PDH transcripts were comparable to blastocysts at other developmental stages (Fig. 4.1A-G). In order to increase the sensitivity of PCR detection, a higher number (45) of PCR cycles was used for amplification of the integrin subunit transcripts. When this was done, low levels of αv, β1 and β3 transcripts could be detected in the 8-10 mm spherical blastocysts, whereas transcripts of other integrin subunits were still not observed (data

not shown). The data in Figure 4.1 also suggest variation in the pattern of up-regulation of this group of integrin subunits between Days 11 and 16 of pregnancy; expression of α3, αv, β1 and β3 transcripts appeared to be progressively up-regulated (Fig. 4.1D-G), in contrast to variable expression of α2 transcripts during this period (Fig. 4.1C). The pattern of expression of α1 transcripts was unique in blastocysts, being undetectable in three spherical stages examined and only observed in elongated blastocysts collected on Days 14 and 16 (Fig. 4.1B). In contrast, expression of α4 and α5 transcripts was not observed in either spherical or elongated blastocysts examined even when a higher number (45) of PCR cycles were used for amplification (data not shown).

B. Expression of Integrin Transcripts in the Placental Tissue and Jag-1 Cells

In the placental tissue (Lane P in Fig. 4.1) collected on Day 28 of pregnancy, expression of α1, α2, α3, αv, β1 and β3 transcripts was observed. The α1, α3, αv and β3 subunits were moderately expressed (Fig. 4.1B, D, E & G), while expression of α2 and β1 transcripts was observed at relatively low levels (Fig. 4.1C & F). In contrast, Jag-1 cells (Lane T in Fig. 4.1) expressed high levels of α1, α2, α3, αv and β3 transcripts (Fig. 4.1B-D, E & G) and low levels of β1 transcripts (Fig. 4.1F). Transcripts for the α5 subunit were detected at low levels in the Jag-1 cells and in the placental tissues (data not shown). Transcripts of α4 subunits were undetectable in either the placental tissues or the Jag-1 cells, although they could be detected in porcine neutrophils (data not shown).

C. Expression of Integrin Transcripts in the Endometrium

G3PDH transcripts could be detected in all endometrial tissues examined (Fig. 4.2A). Four distinct expression patterns of integrin transcripts were observed in porcine

endometrial tissues obtained between Day 3 and 28 of pregnancy. Firstly, relatively constant and high levels of expression for αv, β1 and β3 transcripts were observed in all endometrial tissues obtained (Fig. 4.2F, G & H). Secondly, high-level and developmentally regulated expression was observed for α1 and α2 transcripts. Transcripts for α1 were low in endometrial tissues obtained between Days 3 and 9 of pregnancy (Fig. 4.2B), and remained relatively high in endometrial tissues obtained between Days 10 and 28 of pregnancy except Day 14 of pregnancy (Fig. 4.2B). Expression of α2 transcripts exhibited a variable pattern in endometrial tissues during the period examined (Fig. 4.2C). Thirdly, low-level modulated expression was observed for α3 and α5 transcripts. Expression levels of α3 transcripts were low in endometrial tissues collected between Days 3 and 11.75 of pregnancy (Fig. 4.2D), transiently increased in endometrial tissues obtained around Day 12 of pregnancy (Fig. 4.2D), and were not detected between Days 12.75 and 28 of pregnancy (Fig. 4.2D). Expression of α5 transcripts was observed at very low levels in endometrial tissues obtained between Days 3 and 7.5 of pregnancy (Fig. 4.2F), and was not detected in endometrial tissues collected between Days 11.75 and 28 of pregnancy (Fig. 4.2F). Fourthly, expression of α4 transcripts was not detectable in any endometrial tissues examined (data not shown).

DISCUSSION

During early mammalian embryogenesis, the segregation and differentiation of extraembryonic cell lineages is extremely important for the embryo to sustain its growth and development. Integrins have been shown to play a critical role in early embryogenesis, implantation and placentation in the mouse (Damsky, Sutherland and

Fisher, 1993). Mouse embryos lacking the β1 subunit gene have been shown to develop normally to the blastocyst stage but fail to implant (Fasser and Meyer, 1995; Stephens et al., 1995). It is generally believed that visceral and parietal (or extraembryonic) endoderm cells both arise from primitive endodermal cells (Gardner, 1983; Rosenstraus, Spadoro and Nilsson, 1983). In porcine blastocysts, primitive endodermal cells first appear on the inner face of the ICM on Day 7 of pregnancy, and some of these cells subsequently migrate along the inner face of the trophoblast to give rise to the extraembryonic endoderm (Stroband, Taverne and v an der Bogaard, 1984). On Day 8, extraembryonic endodermal cells have been observed on the inner face of the trophoblast in expanding blastocysts (Richoux et al., 1989). It is probable that the migratory activity of extraembryonic endodermal cells is very high in the expanding spherical blastocysts of 1-2 mm in diameter collected on Day 9 in the present study. Extracellular deposition of fibronectins (FN) was shown to be abundant on the whole inner face of the trophoblast during, and even before, the extraembryonic endodermal migration (Richoux et al., 1989). It is known that FN is a ligand for α3β1, αvβ1 and αvβ3 integrins (Aplin, 1997). Therefore, it is not surprising that high levels of transcripts have been observed for α2, α3, αv, β1 and β3 in these spherical blastocysts. These subunits could potentially form α2β1, α3β1, αvβ1 and αvβ3 functional heterodimers. These data suggest that extraembryonic endodermal migration in porcine blastocysts might be mediated through interactions between integrins α3β1, αvβ1 and αvβ3 possibly expressed on the cell surface of endodermal cells and FN ligands present on the whole inner face of the trophoblast. In contrast, it is interesting to note in the present study that expression of α2, α3, αv, β1 and β3 transcripts was dramatically down regulated in spherical blastocysts

(5-6 mm and 8-10 mm in diameter). These data suggest that cellular migratory activities might be low at these two developmental stages. Indeed, previous morphological observations have shown that the extraembryonic endoderm layer is almost completely established in spherical blastocysts at these developmental stages while the development of extraembryonic mesoderm has still not been initiated (Geisert et al., 1982a; Richoux et al., 1989; Gupta, Bazer and Jaeger, 1996). Therefore, there might be no need for blastocysts at these developmental stages to express high levels of integrin transcripts.

Upon reaching a size of about 10 mm in diameter around Day 11.5 of pregnancy, porcine blastocysts undergo a rapid transition from spherical to elongated forms, which is coincident with the differentiation and initial migration of extraembryonic mesoderm (Geisert et al., 1982a; Gupta, Bazer and Jaeger, 1996). During this initial elongation process, physiological activities of blastocysts are increased as seen by the onset of embryonic estrogen and protein synthesis (Gadby, Heap and Burton, 1980; Godkin et al., 1982; Fischer, Bazer and Fields, 1985). These observations are extended by our findings that expression of α2, α3, αv, β1 and β3 transcripts increase dramatically in elongated blastocysts obtained between Days 11 and 16. Our data on the expression of αv, β1 and β3 transcripts in blastocysts are consistent with the previous report of the expression of αv, β1 and β3 proteins in the trophoblast and implantation sites between Days 11 to 15 of pregnancy (Bowen, Bazer and Burghardt, 1996). Our temporal expression profiles of β1 transcripts in blastocysts are also consistent with a previous study, in which expression of β1 transcripts tended to increase from spherical to filamentous blastocysts obtained from Day 10 to 12 of pregnancy (Yelich, Pomp and Geisert, 1997a). High levels of α3 transcripts detected in the present study might be

mainly expressed in endodermal cells, since α3 proteins were only localized in extraembryonic endodermal cells but were not seen in trophoblast cells of blastocysts obtained between Day 11 to 15 of pregnancy (Bowen, Bazer and Burghardt, 1996).

Two essential processes occur in porcine blastocysts between Day 11 to 16 of pregnancy: the differentiation and migration of extraembryonic mesodermal cells and the initial attachment between the trophoblast and the uterine epithelium (Geisert et al., 1982a; Pusateri et al., 1990; Gupta, Bazer and Jaeger, 1996; Wilson and Ford, 1997). Cell migration requires a reduction in cell contacts, and is generally accompanied by a loss of expression or function of cell adhesion molecules. Changes in the level of α2β1 expression is correlated with the initiation of tumor cell migration in many breast cancers (Zutter and Santoro, 1998; Alford, Pitha-Rowe and Taylor-Papadimitriou, 1998). High levels of the α2β1 integrin ligand, collagen type IV, was shown to be present at the basal laminae of both the trophoblast and the uterine epithelium during early pregnancy in the pig (Bowen, Bazer and Burghardt, 1996). In the present study, expression of α2 transcripts has been shown to be regulated in blastocysts obtained between Days 9 and 16 of pregnancy, which is temporally associated with extraembryonic endodermal and mesodermal cell migration in the pig. In addition, the fluctuation of α2 transcript levels is also observed in porcine endometrial tissues obtained during early gestation in the present study. Taken together, these results suggest that α2β1 integrin might play an important role in the differentiation, proliferation and migration of both extraembryonic lineages and the uterine epithelium during early gestation in the pig.

Attachment between the trophoblast and the uterine epithelium starts on Day 13 to 14 of pregnancy in the pig (Dantzer, 1985). The present study demonstrates that

expression of α_1 transcripts is not detected in porcine blastocysts until Day 14 of pregnancy. Furthermore, expression levels of α_1 transcripts were very high in Day 28 placental tissue and in trophoblast cells (Jag-1) derived from Day 14 blastocysts. It is also interesting to note in the current study that expression of α_1 transcripts was higher in endometrial tissues obtained at the time of embryo-uterine contact compared to other developmental stages. These results extend previous observations on the contact-inductive nature of α_1 expression during embryo implantation in humans and mice, where α_1 expression on the trophoblast is a response to trophoblast contact with ECM in the uterus (Damsky, Fitzgerald and Fisher, 1992; Sutherland, Calarco and Damsky 1993). Integrin $\alpha_1\beta_1$ is a known receptor for collagen and laminin (Forsberg et al., 1990; Lessey, 1997). However, the α_1 proteins, as well as type IV collagen and laminin were demonstrated to localize only at the basal laminae of both the uterine epithelium and the trophoblast at implantation sites in the pig (Bowen, Bazer and Burghardt, 1996; Burghardt et al., 1997). As such, $\alpha_1\beta_1$ integrin would not be appropriately positioned to participate in adhesive interactions between the apical surfaces of the trophoblast and the uterine epithelium (Burghardt et al., 1997). However, the integrin $\alpha_1\beta_1$ might play a role in stabilizing the adhesion interactions between the trophoblast and its underlying basement membrane (BM) and between the uterine epithelium and its BM, respectively. Intact and firm trophoblast and uterine epithelial layers are essential for supporting the subsequent extraembryonic mesodermal migration and the extensive morphological remodeling in blastocysts and the endometrium, during the attachment/ implantation process in the pig, such as the formation of trophoblastic caps and endometrial protrusions (Dantzer, 1985; Keys and King, 1990). Furthermore, expression of α_1

transcripts was not detected in small spherical blastocysts (1-2 mm in diameter) in the present study, which suggests that integrin $\alpha_1\beta_1$ is not likely to be involved in the extraembryonic endodermal cell migration in the pig. Integrin $\alpha_3\beta_1$ is a cell surface receptor for collagen, laminin and FN (Lessey, 1997), all of which have been shown to be present in the ECM underlying the trophoblast (Richoux et al., 1989; Bowen, Bazer and Burghardt, 1996; Burghardt et al., 1997). Unlike the α_1 subunit, expression of α_3 transcripts was detected in the present study in small spherical blastocyst (1-2 mm in diameter). In addition, expression of α_3 transcripts increased in elongated blastocysts obtained between Days 11 and 16, which correlates with the initiation and migration of extraembryonic mesoderm in the pig. However, expression of α_3 proteins was restricted to the basal lamina of the uterine epithelium, and was detected in the endoderm but not in the trophoblast of blastocysts during early pregnancy in the pig (Bowen, Bazer and Burghardt, 1996). Taken together, we hypothesize that integrin $\alpha_3\beta_1$ might be directly involved in extraembryonic endodermal and mesodermal cell migration in the pig.

In the present study, similar expression patterns of β1 and β3 transcripts were observed both in the placental tissues at Day 28 and in Jag-1 cells, with β3 levels being higher in both cases. In contrast, Bowen and Hunt (1999) showed that expression of β1 at the mRNA and protein levels was higher than that of β3 in mouse placenta, which is known to be invasive during embryo implantation. In addition, expression of the β1 subunit was shown to be up regulated during trophoblast invasion in human uterus (Aplin, 1997). Furthermore, β1-knockout mouse embryos develop normally to the blastocyst stage but fail to sustain implantation (Fassler and Meyer, 1995; Stephens et al., 1995). Collectively, those results are consistent with a role for β1 integrins in the invasive

type of implantation seen in the human and mouse. In contrast, low-level expression of the β1 subunit in the porcine placenta might contribute to the non-invasive type of embryo implantation seen in the pig. High-level expression of β3 transcripts observed in the present study might be required for the adhesion between trophoblast cells in porcine blastocysts, which is supported by a previous observation by Bowen, Bazer and Burghardt (1996) that β3 proteins were restricted to the intercellular borders between trophoblast cells. Therefore, an intact layer of trophoblast cells could be maintained to ensure the integrity of the non-invasive epitheliochorial placenta in the pig.

The high-level expression of α_v, β_1 and β_3 transcripts observed in endometrial tissues during early gestation in the present study is consistent with the previous observation of α_v, $\beta_1$and β_3 protein expression in the uterine epithelium by Bowen, Bazer and Burghardt (1996). Integrin $\alpha_v\beta_3$ is a receptor for both FN and vitronectin (VN) (Hynes, 1992; Aplin, 1997). Bowen, Bazer and Burghardt (1996) demonstrated that both FN and VN were present on apical surfaces of the trophoblast and the uterine epithelium during early pregnancy in the pig. In addition, they found that both α_v and β_3 subunits were localized on the apical surface of the uterine epithelium during early pregnancy and at implantation sites, but were restricted to intercellular borders between trophoblast cells between Days 11 to 15 of pregnancy in the pig. These results suggest that interactions between the integrin $\alpha_v\beta_3$ on the uterine epithelium and its ligands (FN and VN) on the apical surface of the trophoblast might play a major role during embryo implantation in the pig. Since conceptus/fetal development in the pig relies on a non-invasive epitheliochorial placenta, firm adhesion between the trophoblast and the uterine

epithelium is essential to establish and sustain an efficient interface for nutrient and waste exchange with the maternal system.

Expression of integrin subunits α_4 and α_5 at the fetal/maternal interface appears to be a species-specific phenomenon. Expression of both α_4 and α_5 subunits was absent in human pre-implantation embryos (Campbell et al., 1995). Expression of α_5 proteins was only localized to the uterine stroma and not the uterine epithelium by Lessey (1994), although a number of discrepancies exist in the literature concerning the endometrial expression of α_5 subunit (Lessey et al., 1992; Tabibzadeh, 1992; Klentzeris et al., 1993). In contrast, expression of α_5 but not α_4 subunits was observed in mouse pre-implantation embryos (Sutherland, Calarco and Damsky, 1993). Furthermore, implantation defects were not observed in mouse embryos lacking α_4 and α_5 (Yang, Rayburn and Hynes, 1993 & 1995), suggesting that they are not involved in the implantation processes. In the present study, α_4 and α_5 transcripts were not detected in blastocysts obtained between Days 9 and 16 using the RT-PCR approach, although expression of α_4 and α_5 could be detected in porcine neutrophils and in Jag-1 cells, respectively, which were used as positive controls. Moderate and intense staining of α_4 and α_5 was previously reported in porcine uterine epithelium and trophoblast between Days 11 to15 of pregnancy and also at attachment points between the uterine epithelium and the trophectoderm (Bowen, Bazer and Burghardt, 1996). Considering the sensitivity of the RT-PCR approach, the transcript levels of α_4 and α_5 subunits detected in the present study are unlikely to produce the high levels of proteins in the uterine epithelium and in the trophoblast observed in the previous study by Bowen, Bazer and Burghardt (1996). Therefore, further clarification of the expression of α_4 and α_5 subunits during early pregnancy in the pig is

needed. In addition, it is obvious that there is a limitation on the number of animals used in the experiment examining integrin expression in blastocysts, although the number of blastocysts is resonably sufficient for representing individual stages of embryo development examined.

In summary, in the present study, the expression of eight integrin subunit (α1, α2, α3, α4, α5, αv, β1, and β3) transcripts was determined in blastocysts during early embryogenesis and in the endometrium during early gestation in the pig. Our data suggest that integrins might play important regulatory roles in early embryogenesis, implantation/attachment and placentation in the pig.

Table 4.1 Blastocsyt and placental tissue samples used in this experiment.

Sample	Day of pregnancy	Number and developmental stage of blastocysts and placental tissue	Number of pigs slaughtered
S1	9	37; Spherical (1-2 mm in diameter)	3
S2	10	19; Spherical (5-6 mm in diameter)	2
S3	10.5	8; Spherical (8-10 mm in diameter)	1
F1	11	8; Elongated blastocysts	1
F2	12	9; Elongated blastocysts	1
F3	14	12; Elongated blastocysts	1
F4	16	6; Elongated blastocysts	1
P	28	Placental tissue	1

Table 4.2 PCR primer sequences and predicted product sizes for integrin subunits#.

Target gene	Primer sequence	Size (bp)	Source*
G3PDH	5' ACCACAGTCCATGCCATCAC 3'	452	Yelich et al., 1997
	5' TCCACCACCCTGTTGCTGTA 3'		
α1	5' CTTCAATGTCTCTGATGAATTGGC 3'	975	AB000470; X68742;
	5' GAAGAGGGCAGCACCACCAAGGC 3'		X52140
α2	5' GGAGGAGACAACTTTCAGATGG 3'	1,206	M28249; Z29987
	5' GGGCAGGGCTAGTGCCAGGG 3'		
α3	5' GACTGGTGCTGTGTACCTGTG 3'	790	M59911; D13867
	5' CCCGCCTGGTTCATGAAGACATA 3'		
α4	5' GCCCTAATGGAGAACCTTGTGG 3'	1,264	L12002; X53176
	5' CTCTGCCTTTCTGTTCACATCC 3'		
α5	5' CTCAGGACTCGTCAGACACCC 3'	881	M74954; M74116;
	5' CCATAGCTGCCTTCTGCCTTGG 3'		X79003
αv	5' GCAGATGTGTTTATTGGAGCACC 3'	1,065	M14648; J02826;
	5' GAGCTCAGCTTCGTAGGCACC 3'		M18365; U14135
β1	5' GACCTCTACTACCTTATGGACC 3'	1,900	X07979; U12309
	5' GGATTTTCACCCGTGTCCCA 3'		Y00769
β3	5' CTACCCTGTGGACATCTACTAC 3'	792	U95204; X72378
	5' GGCATTGAAGGACAGCGACAGCTC 3'		M35999; M20311

#All primer sequences were listed from 5' end to 3' end. * Primers were designed according to conserved regions of human and mouse cDNA sequences obtained from GenBank database unless indicated. GenBank access numbers or references were listed.

Figure 4.1 RT-PCR amplification of G3PDH (A) and integrin subunits α_1 (B), α_2 (C), α_3 (D), α_v (E), β_1 (F) and β_3 (G) in blastocysts, placental tissues and Jag-1 cells. Total RNA was extracted from blastocysts from gilts representing Days 9 (S1), 10 (S2), 10.5 (S3), 11 (F1), 12 (F2), 14 (F3), and 16 (F4) of pregnancy, respectively, placental tissues of Day 28 (P) and trophoblast cells (Jag-1) (T). M, 100 bp DNA ladders. N, negative control. Names and predicted sizes of target genes are indicated to the right and left sides of the figures, respectively. PCR products were run in 1.2% (w/v) agarose gel, and gels were stained with ethidium bromide.

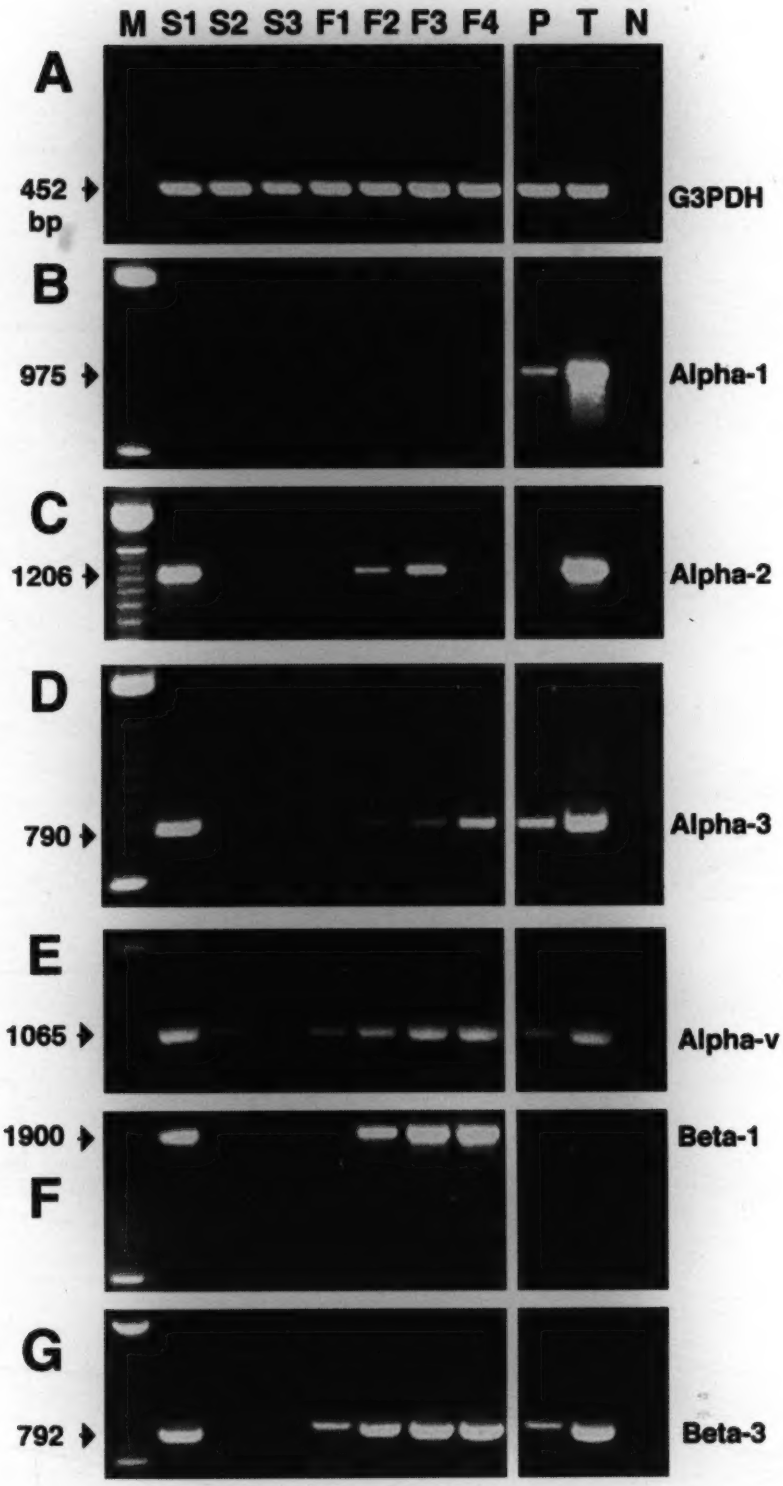
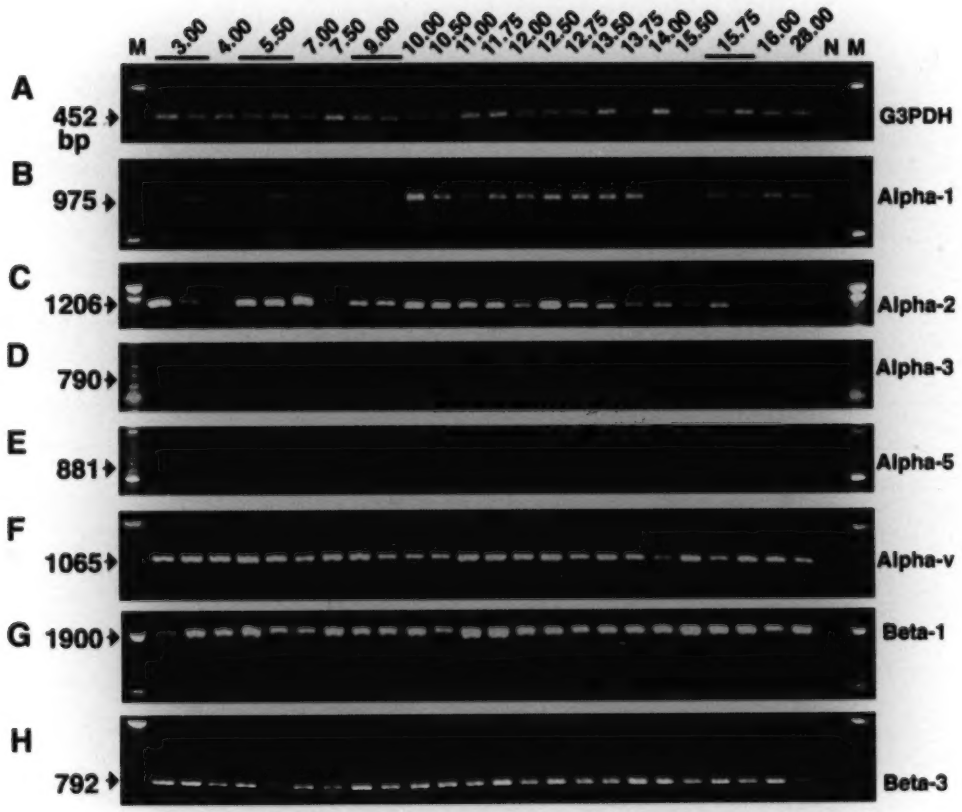

M S1 S2 S3 F1 F2 F3 F4 P T N
A
452 bp
G3PDH
B
975
Alpha-1
C
1206
Alpha-2
D
790
Alpha-3
E
1065
Alpha-v
1900
Beta-1
F
G
792
Beta-3

Figure 4.2 RT-PCR amplification of G3PDH (A), α_1 (B), α_2 (C), α_3 (D), α_5 (E), α_v (F), β_1 (G) and β_3 (H) in the endometrium during early gestation. Total RNA was extracted from endometrial tissues obtained from individual gilts on Days 3, 3, 4, 5.5, 5.5, 7, 7.5, 9, 9, 10, 10.5, 11, 11.75, 12, 12 .5, 12.75, 13.5, 13.75, 14, 15.5, 15.75, 15.75, 16 and 28 of pregnancy, respectively. M, 100 bp DNA ladders. N, negative control. Names and predicted sizes of target genes are indicated to the right and left sides of the figures, respectively. PCR products were run in 1.2% (w/v) agarose gel, and gels were stained with ethidium bromide.


M
3.00
4.00
5.50
7.00
7.50
9.00
10.00
10.50
11.00
11.75
12.00
12.50
12.75
13.50
13.75
14.00
15.50
15.75
16.00
28.00
N
M
A
452 bp
G3PDH
B
975
Alpha-1
C
1206
Alpha-2
D
790
Alpha-3
E
881
Alpha-5
F
1065
Alpha-v
G
1900
Beta-1
H
792
Beta-3

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CHAPTER 5

GENERAL DISCUSSION AND CONCLUSIONS

Considerable embryonic losses occur from Days 13 to 20 of pregnancy in the pig, which is coincident with mesodermal migration in blastocysts, blastocyst elongation and the trophoblast-uterine epithelium attachment (implantation). Little is known about the mechanisms regulating these processes in the pig, although great progress has been made in understanding molecular and cellular processes during embryo implantation and placental development in the mouse during the last decade (Schultz and Edwards, 1997). Therefore, there is a need to characterize the expression of important factors in the embryo and in the uterus during early gestation, especially during the peri-implantation period, in the pig. To date, a variety of soluble and membrane-associated factors that are relevant to the implantation process in other species have been shown to be present in blastocysts and the uterus during the peri-implantation period in the pig (Geisert and Yelich, 1997; Burghardt et al., 1997).

In the first experiment of the present study, we extended earlier findings of Ramsoondar et al. (1993) in which a porcine trophoblast cell line (Jag-1) was found to secrete growth factor(s) stimulating the proliferation of porcine macrophages. In the present study, serum-free conditioned media of Jag-1 exhibited a stimulatory effect on the proliferation of porcine macrophages in a dose-dependent fashion. However, a specific anti-CSF-1 hybridoma supernatant YY106 was able to abrogate almost completely this stimulatory activity. This result strongly suggests that CSF-1 is the major growth factor secreted by JAG-1 trophoblast cells, and is responsible for the observed bioactivity. Subsequently, the coding sequence of a porcine CSF-1 cDNA was

characterized, which showed significant homologies with human and mouse CSF-1 cDNA sequences. In addition, the CSF-1 gene was demonstrated to be present as a single copy in the pig genome, which extended earlier observations on the CSF-1 gene in human and mouse (Kawasaki et al., 1985; Ladner et al., 1987; Rajavashisth et al., 1987). This information provides the basis for further studies on the regulation of CSF-1 expression in the pig and its role in conceptus development.

Growth factors and cytokines are believed to play important roles in regulating cellular proliferation, differentiation and morphogenesis during mammalian embryonic development. They can function in an autocrine fashion within the embryo and/or in a paracrine fashion through interactions between the embryo and the uterus (Kaye PL, 1997; Kaye and Harvey, 1995; Schultz and Heyner, 1993). Some growth factors and cytokines have been demonstrated to be critical to implantation in mammals (Nie et al., 1997, Polan et al., 1995; Harvey et al., 1995). For example, a lack of endometrial expression of LIF could result in the failure of embryo implantation in the mouse (Stewart et al., 1992; Shellard et al., 1996). A number of growth factors and cytokines have been shown to be present in blastocysts and the uterus during the peri-implantation period in the pig, although little or no information exists at this point regarding their functions (Geisert and Yelich, 1997). The expression of CSF-1 at both the mRNA and protein level has been reported previously in porcine uterine, conceptus, allantochorionic and fetal tissues during the post-implantation stages of pregnancy by Tuo et al. (1995). In order to understand the role of CSF-1 during early embryonic development and implantation in the pig, the expression of CSF-1 and its receptor (c-fms) transcripts was determined in blastocysts and the endometrium during early gestation. In contrast to an

earlier study in which expression of c-fms but not CSF-1 was detected in pre-implantation embryos in the mouse (Arceci et al., 1992), expression of both c-fms and CSF-1 transcripts were detected in both blastocysts and the endometrium during the peri-implantation period in the present study. This result suggests that CSF-1 might play both paracrine and autocrine roles in embryonic development during early gestation in the pig. It has been demonstrated in mice and humans that CSF-1 is a growth factor involved in trophoblast proliferation and differentiation (Guilbert et al., 1991; Pollard et al., 1987). Therefore, we speculate that both endometrial and embryonic expression of CSF-1 could be directly involved in the proliferation and differentiation of porcine trophoblast cells, which is essential for the rapid expansion and elongation of blastocysts during pre-implantation development in the pig. A low level of c-fms expression in blastocysts prior to implantation might partially contribute to the non-invasiveness of porcine trophoblast cells during pregnancy.

The roles of MMPs and TIMPs in tissue remodeling during embryo implantation have been extensively studied in rodents and humans (Damsky et al., 1997; Bass et al., 1997; Alexander et al., 1996). A large amount of convincing evidence indicates that trophoblast-derived MMP-9 is responsible for the invasive type of embryo implantation seen in humans and rodents through its ability to degrade the basement membrane of the endometrium. In the second experiment of the present study, MMP-9 expression has not been detected by RT-PCR, which is in agreement with a previous study by Menino et al. (1997). These data indicate that a lack of MMP-9 expression by blastocysts during the peri-implantation period might partially explain the non-invasive type of implantation seen in pigs (Fig. 5.1). This observation provides further indirect

evidence for the essential role of MMP-9 in embryo implantation in mammals with invasive types of placentation (Salamonsen, 1999). Low levels of MMP-2 and MMP-9 transcripts were detected in Jag-1 trophoblast cells by RT-PCR, and significant levels of proMMP-2 and proMMP-9 were shown by zymography in the present study. Interestingly, Jag-1 cells were able to penetrate a layer of Matrigel artificial basement membrane in an *in vitro* invasion chamber model system, (Chai et al., unpublished observations). These observations provide an explanation at the molecular level for previous histological observations on the invasiveness of porcine embryonic trophoblast cells at ectopic sites (Samuel and Perry, 1972). The differential behavior of porcine trophoblast cells under *in vivo* and *in vitro* conditions suggests that negative regulatory factor (s), possibly present in the uterine fluid and/or induced within the blastocyst itself, might suppress the transcription of the MMP-9 gene in trophoblast cells of blastocysts.

Cell migration and interactions during early embryogenesis require the involvement of MMPs, as demonstrated in the mouse (Behrendtsen and Werb, 1997). In the second experiment of the present study, co-expression of MMP-2, TIMP-2 and MMP-14 transcripts were observed in blastocysts during the peri-implantation period. In addition, proMMP-2 levels in uterine flushings of pregnant gilts were elevated during this period. TIMP-2 expression was observed in all type of cells in porcine blastocysts at the time of embryo-uterine contact (Menino et al., 1997). Collectively, these results indicate that the MMP-2/TIMP-2/MMP-14 system might contribute to the spatially regulated extracellular proteolysis required for the extraembryonic mesoderm migration in porcine blastocysts (Fig. 5.1). This is consistent with the proposed role of this system in murine embryogenesis (Apte et al., 1997). Demonstrated expression of the MMP system in

porcine endometrium in our study suggests that extensive endometrial tissue remodeling occurs to prepare the endometrium for embryo implantation in the pig. The preliminary observations of the expression of other MMPs and MMP regulators, obtained in the present study, should pave the way for further examination of the functions of these factors during embryo development and implantation in the pig.

Mammalian early embryogenesis and implantation involve extensive cell-cell and cell-extracellular matrix interactions. These cellular interactions are known to be mediated by several classes of cell surface adhesion molecules, the most important of which are the integrins (Aplin, 1997; Lessey, 1997; Gille and Swerlick, 1996; Damsky et al., 1993; Hynes, 1992). Using indirect immunocytochemical techniques, some integrin proteins were previously localized in porcine uterine epithelium and trophoblast *in vivo* and *in vitro* by Bowen et al. (1996 and 1997). In order to understand the role of integrins during embryonic development in the pig, the third experiment of the present study was taken to examine the expression patterns of transcripts for eight integrin subunits (α1, α2, α3, α4, α5, αv, β1 and β3) in blastocysts and the endometrium during early gestation using an RT-PCR approach. Expression of α2, α3, αv, β1 and β3 transcripts in blastocysts correlates with the extraembryonic endodermal and mesodermal migration processes during early embryogenesis in the pig. Expression of α1, α2, α3, αv, β1 and β3 transcripts have been observed in Jag-1 cells and Day 28-placental tissue. These findings suggest important roles for integrins in the processes of early embryogenesis, embryo implantation and placentation in the pig. Based on the present data, in conjunction with previous studies, a hypothetical role of integrins during early

Table 5.1 Cellular regulation of integrin, MMPs and TIMPs expression by growth factors/cytokines

Experimental cell System	Gene Regulated	Regulator	Reference
Human dermal fibroblasts	α1 ↓; α5 ↑	PDGF	Gailit et al., 1996
Human breast and esophageal cancer cells	α2 ↑; α3 ↑	HBGF	Narita et al., 1996; Sato et al., 1996
Chick neuroepithelial cells	α6 ↑	IGF-I	Frade et al., 1996
Human endothelial cells	α1 ↑; α2 ↑	VEGF	Senger et al., 1997
Human salivary gland duct cells and rat mesangil cells	β1 ↑	TGF-β1	Azuma et al., 1996; Kagami et al., 1996
Human dermal fibroblasts	α1 ↑; α5 ↑	TNF-α; INF-γ; IL-1	Gailit et al., 1996
Human endometrial stromal cells	TIMP-1 ↑; TIMP-3 ↑	IL-1	Huang et al., 1998
Human normal and maligmant keratinocytes and ovarian cancer cells	MMP-9 ↑	EGF	Charvat et al., 1998; Elleroek et al., 1998
Human uterine cervical fibroblasts	MMP-3 ↑; TIMP-1 ↑	EGF	Hosono et al., 1996
Keratinocytes; kidney and mammary epithelial cells	TIMP-3 ↑	HGF	Castagnino et al., 1996
Lung cancer cells	MMP-2 ↑	IGF-I	Long et al., 1998
Human breast cancer cells	MMP-9 ↑	EGF; Amphiregulin	Kondapaka et al., 1997
Human prostatic cancer cells	MMP-2 ↓; TIMP-1 ↑	IL-4; IL-10	Wang et al., 1996
Human prostatic cancer cells	MMP-2 ↑; MMP-9 ↑	TGF-β1	Sehgal & Thompson et al., 1999
Human vascular smooth muscle cells	MMP-1 ↑; MMP-3 ↑; MMP-9 ↑	VEGF	Wang & Keiser, 1998

embryogenesis and implantation in the pig is shown schematically in Figure 5.2. The αv subunit was found on the apical surface of the uterine epithelium but not of the trophoblast during early pregnancy in the pig by Bowen et al. (1996). This suggests that endometrial integrins αvβ1 and αvβ3 might play a critical role in the attachment between the trophoblast and the uterine epithelium. Embryonic integrins αvβ1 and αvβ3 could instead facilitate the extraembryonic endodermal and mesodermal cell migration. A lack of strong expression of the β1 subunit in trophoblast cells in the pig is consistent with the critical role of β1 integrins in the highly invasive implantation seen in human and mouse.

Molecular interactions at the embryo-maternal interface during early pregnancy in mammals appear to be mediated through embryonic cytokines (Reviewed by Simon et al., 1998 and Martal et al., 1997). Harvey et al. (1995) showed that LIF and EGF could stimulate the secretion of uPA and MMP-9 in day-7 mouse blastocysts during their outgrowth *in vitro*. Recently, a number of growth factors/cytokines have been shown to regulate the expression and activity of integrins, MMPs and TIMPs in many types of normal and malignant cells (Table 5.1). As an example, IL-1β was shown to both increase the expression of MMP-9 and decrease the expression of TIMP-1 and TIMP-3 in human endometrial stromal cells (Huang et al., 1998). In contrast, the same authors also demonstrated that TGF-β augmented the expression of TIMP-1 and TIMP-3 but did not affect MMP-9 expression. As a result, it was of interest to know whether other growth factors/cytokines are expressed during early conceptus development in the pig. RT-PCR approaches have been used extensively in measuring the expression of cytokines in porcine immune cell populations (Dozois et al., 1997; Reddy et al., 1996; Vezina et al., 1995). Therefore, an additional experiment was carried out as a preliminary survey on the

expression of 9 cytokines, IL-1, IL-2, IL-4, IL-6, IL-8, IL-10, TNF-α, TNF-β and IFN-γ, during early conceptus development in the pig (Table 5.2 and Appendix A). Our preliminary data indicates that expression of IL-1, IL-6, IL-8 and IFN-γ is developmentally regulated in blastocysts during the peri-implantation period (Fig. 5.3). However, expression of IL-2, IL-4, IL-10, TNF-α and TNF-β was not observed in blastocysts at any of the developmental stages examined, or in the Jag-1 cells, or in Day 28 placental tissue (Fig. 5.3).

Interestingly, two IFN-γ cDNA fragments of 378 bp and 300 bp were amplified in both spherical blastocysts of 8-10 mm in diameter (Panel S3, Fig. 5.3) and all elongated blastocysts (Panels F1 to F4, Fig. 5.3), but not in the two earlier spherical stages (Panels S1 & S2, Fig. 5.3). This result is in agreement with previous studies by Ramsoondar (1994) and Lefevre et al. (1990). Both studies showed that two species of INF-γ mRNA with sizes of approximately 1. 4 kb and 1.3 kb, respectively, were present in Day 14 to 17 blastocsyts. It is likely that cDNA fragments of 378 bp and 300 bp were derived from IFN-γ mRNA species of 1. 4 kb and 1.3 kb, respectively. Furthermore, two species of IFN-γ mRNA exhibited different patterns of expression in blastocysts. Progressively increased expression was observed for the IFN-γ mRNA represented by the 378-bp cDNA fragment as blastocysts developed from spherical to elongated shapes (Panels S1 to F4, Fig. 5.3). In contrast, expression of the IFN-γ mRNA represented by the 300-bp cDNA fragment first appeared and was dominant in spherical blastocysts of 5-6 mm in diameter, and decreased as blastocyts elongated (Panels S3 to F4, Fig. 5.3). Expression of IFN-γ transcripts was not detected in Jag-1 cells (Panel T, Fig. 5.3),

although low expression of the IFN-γ mRNA represented by the 378-bp cDNA fragment was observed in Day-28 placental tissue (Panel P, Fig. 5.3).

Complex relationships between growth factors/cytokines, integrins, MMP/ TIMP and extracellular matrix have been realized during normal and abnormal cellular processes. For example, the interaction between epidermal growth factor (EGF) and integrin α3β1 has been shown to contribute to migratory and invasive properties of gliomas (Tysnes et al., 1997). Collaboration between platelet-derived growth factor (PDGF) receptor β and integrin α3β1 was shown to be required for the motile response of human mesothelioma cells to PDGF BB *in vitro* Klominek et al., 1998), as was the interaction between insulin-like growth factor-I (IGF-I) receptor and integrin αvβ3 necessary for the cellular response of vascular smooth muscle cells to IGF-I (Zheng and Clemmons, 1998). Furthermore, αvβ1 can function as a receptor for latent TGF-β, which might facilitate the role of latent TGF-β in the ECM (Munger et al., 1998). The interaction between latent TGF-β and αvβ1 may localize latent TGF-β to the surface of specific cells and allow the TGF-β gene product to initiate signals by both TGF-β receptor- and integrin-mediated pathways. In addition, a functional cooperation between IGF-I receptor and integrin αvβ5 was shown to be required for pulmonary metastasis of multiple tumor cell types *in vivo* (Brooks et al., 1997). Activation of both IGF-I receptor and integrin α5β1 is required for IGF-I to stimulate wound healing on rabbit ears (Galiano et al., 1996). A more intriguing observation is that the focal adhesion kinase p125 (FAK), a cytosolic tyrosine kinase, mediates signal transduction of not only integrins but also tyrosine kinase receptors including PDGF, HGF, insulin and IGF-I receptors (Baron et al., 1998). Therefore, as a member of the tyrosine kinase receptor

family, c-fms might be able to mediate cellular processes, alone or in collaboration with integrins and MMPs, during implantation and early embryogenesis in the pig. This hypothesis needs to be verified by further functional studies of porcine CSF-1. In relation to porcine implantation, an immediate study that could be carried out is to examine the role of CSF-1, IL-1, IL-6, IL-8 and IFN-γ in regulating the expression of trophoblastic integrins and/or MMPs and their regulators in Jag-1 cells.

MMP-2 and integrin αvβ3 were co-localized on angiogenic blood vessels and melanoma cells *in vivo*, and expression of αvβ3 on cultured melanoma cells enabled these cells to bind MMP-2 in a proteolytically active form, facilitating directed cellular invasion *in vitro* (Brooks et al., 1996). Antibodies against α3 and α2 integrin subunits could induce the activated form of MMP-2 and enhance proMMP-2 secretion and thereby facilitate cell invasion through reconstituted basement membrane (Matrigel) by human rhabdomyosarcoma cells *in vitro* (Kubota et al., 1997). Integrin α2β1 has been shown to be a positive regulator of MMP-1 and type I collagen expression by osteogenic cell lines (Riikonen et al., 1995). Integrin ligation could regulate the expression of cytokine receptors on the cell surface of polymorphonuclear leukocytes (PMN) through signaling pathways involving protein tyrosine kinases ("outside-in" signalling), thereby influencing the chemotaxis of PMN into the interstitium (Simms et al., 1997; Hynes, 1992).

As our knowledge accumulates on the regulation and interactions between growth factors/cytokines, MMPs and integrins, it should be possible to decipher in detail the molecular mechanisms regulating implantation and early embryogenesis in the pig. As a result, approaches could be developed to minimize or prevent high embryonic loss

occurring during this period of development in order to improve further reproductive efficiency in this important domestic animal.

Figure 5.1 Schematic representation of the role of matrix matelloproteinase (MMP) system in implantation and early embryogenesis in the pig. The lack of expression of MMP-9 in the blastocyst and the co-localization of MMP-2 and TIMP-2 in the uterine lumen are consistent with the formation of the non-invasive type of placentation in the pig. The co-expression of the tri-molecular complex, MMP-2/TIMP-2/TIMP-14, in blastocysts would facilitate cell migration during mesodermal development in the pig.

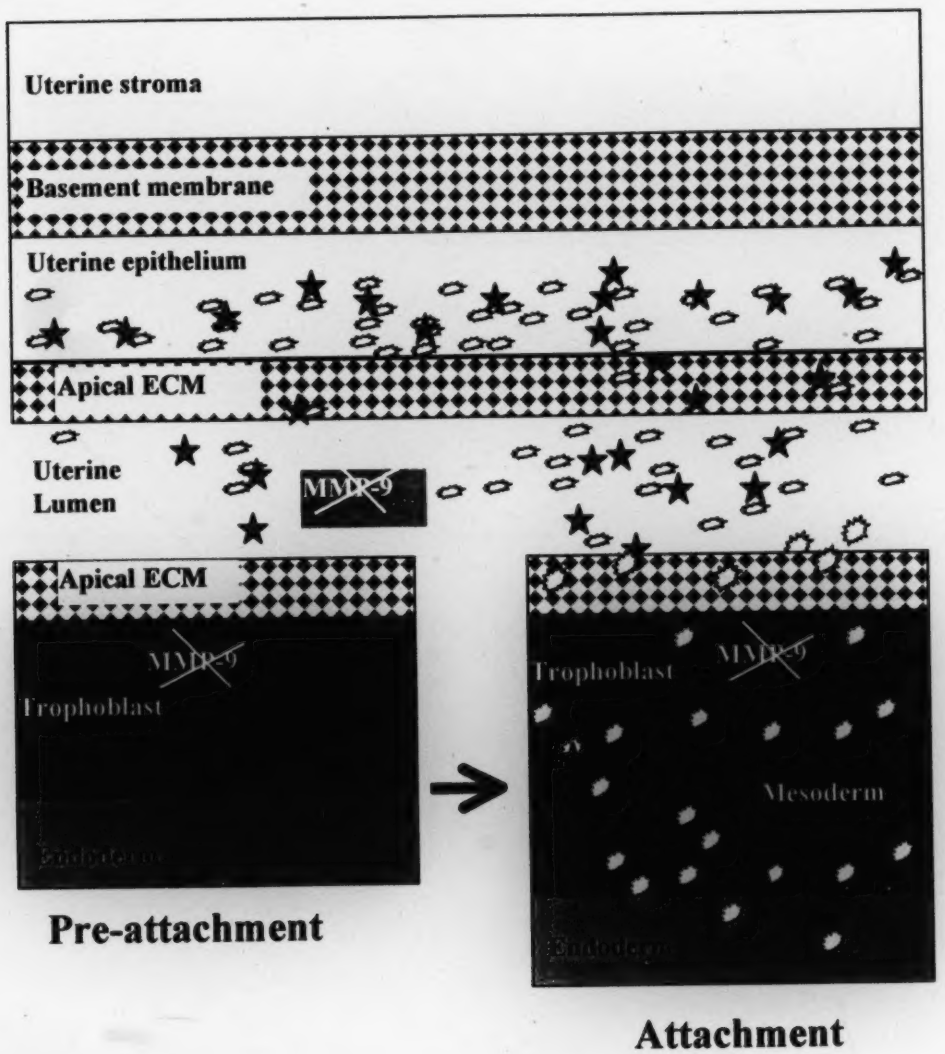

Uterine stroma
Basement membrane
Uterine epithelium
Apical ECM
Uterine
Lumen
MMP-9
Apical ECM
MMP-9
Trophoblast
Trophoblast
MMP-9
Mesoderm
Pre-attachment
Attachment
Type IV collagen
(Substrate of MMP-2)
Fibronectin
(Substrate of MMP-2)
MMP-2
TIMP-2
Vitronectin

Figure 5.2 Schematic representation of the hypothetical role of the integrin family in early embryogenesis and implantation in the pig. An absence of αv subunits at the apical surface of the trophoblast during early pregnancy suggests that endometrial integrins αvβ1 and αvβ3 might play a critical role in the attachment between the trophoblast and the uterine epithelium. Embryonic integrins αvβ1 and αvβ3 could facilitate the extraembryonic endodermal and mesodermal cell migration. A lack of strong expression of the β1 subunit in trophoblast cells in the pig with a non-invasive type of implantation is consistent with the critical role of β1 integrins in the invasive implantation seen in human and mouse.

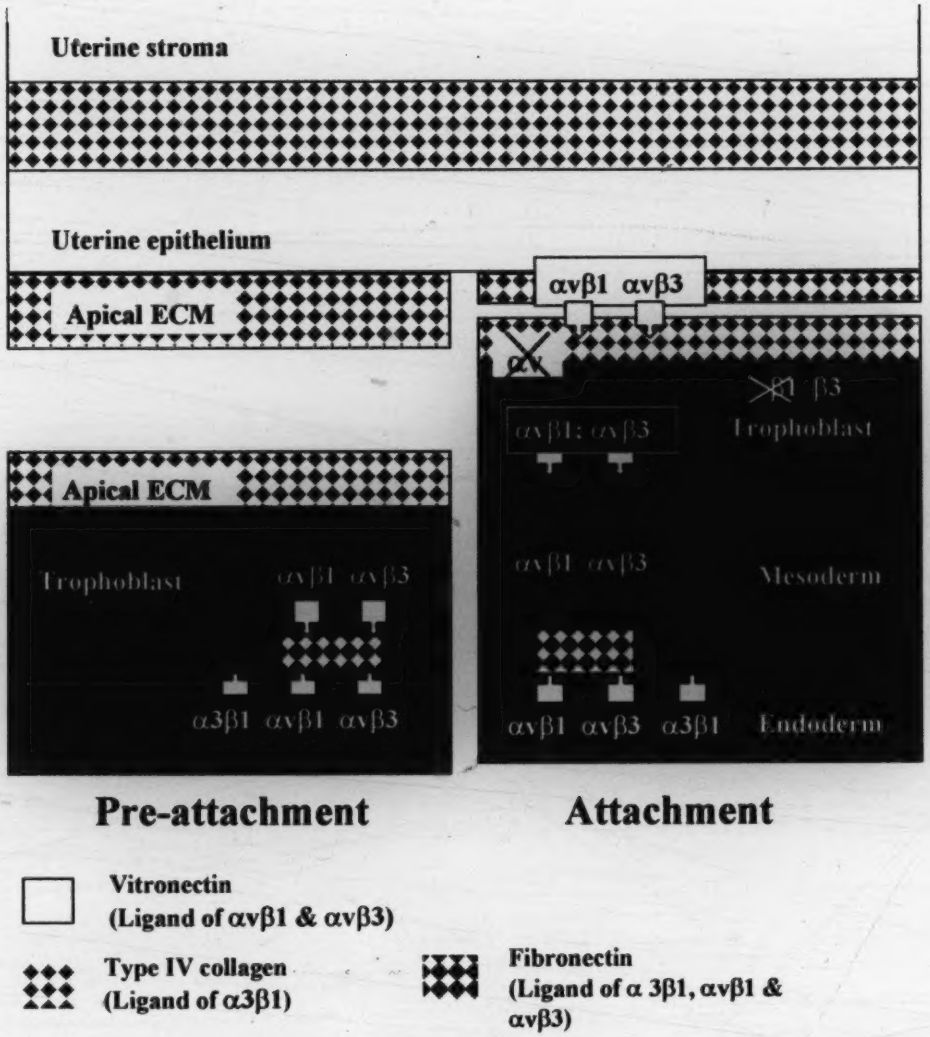
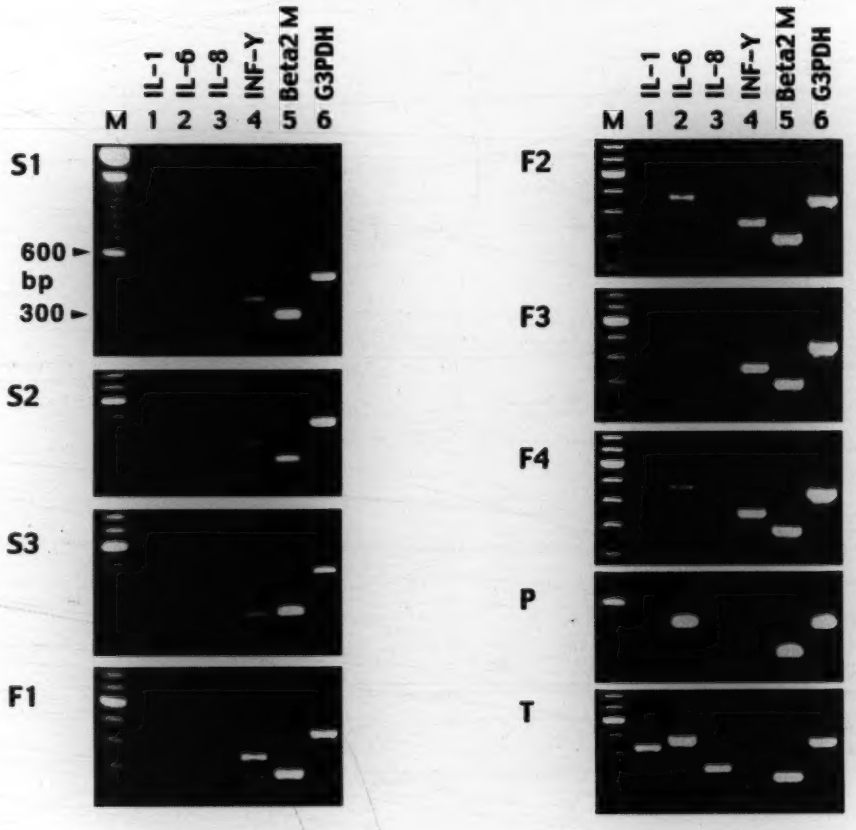


Figure 5.2 Schematic representation of the potential role of the integrin family in the pig.

Figure 5.3 Characterization of the expression for IL-1 (Lane 1), IL-6 (Lane 2), IL-8 (Lane 3), and IFN-γ (Lane 4) transcripts in blastocysts during the peri-implantation period in the pig. Expression of IL-2, IL-4, IL-10, IL-12, TNF-α and TNF-β transcripts was not detected at all stages of blastocyst development examined. M, 100-bp DNA ladder.


M
IL-1
IL-6
IL-8
INF-Y
Beta2 M
G3PDH
1
2
3
4
5
6
S1
600
bp
300
S2
S3
F1
F2
F3
F4
P
T

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APPENDIX

EXPRESSION OF CYTOKINES IN BLASTOCYSTS DURING PERI-IMPLANTATION DEVELOPMENT IN THE PIG

MATERIALS AND METHODS

A. Animals

Sexually mature gilts (PIC Camborough) of similar age and weight, and two recorded estrous cycles, were heat-checked three times daily (08:00, 16:00 and 24:00) for onset of estrus (standing heat) with a vasectomized boar. At 16 and 24 hours after onset of estrus, gilts were artificially inseminated using fresh-pooled crossbred semen. All animals were handled in accordance with the Canadian Council on Animal Care (1993). Pregnant gilts were slaughtered, and the reproductive tracts were recovered within 10 minutes of death.

B. Collection of Blastocysts, Placental Tissues and Jag-1 cells

Uterine horns were excised at slaughter and individually flushed with physiological saline. Blastocysts were collected from the uterine flushings and rinsed briefly in physiological saline to remove uterine debris. Spherical blastocysts were classified according to their sizes into three groups: 1-2 mm (n=37), 5-6 mm (n=19) and 8-10mm (n=8) in diameter, which were collected from gilts on Days 9 (n=3), 10 (n=2) and 10.5 (n=1) of pregnancy, respectively (Table 4.1). The number of elongated blastocysts obtained from each pig was estimated by counting the free bulbous ends of aggregated blastocysts. Sections of placental tissue were obtained from the allantochorial membrane distal to the embryonic region from Day 28 conceptuses. A porcine

trophoblast cell line, Jag-1, was included for comparison of gene expression, and Jag-1 cells were cultured and harvested as described previously by Ramsoondar et al. (1993). All tissue and cell samples were immediately snap-frozen in liquid nitrogen after collection and stored at –80 °C.

C. RNA Extraction

Total cellular RNA was extracted using TRIzol reagent according to the manufacturer's instructions (Gibco/BRL, Gaithersburg, MD, USA). TRIzol reagent was directly added to the frozen embryonic tissues or Jag-1 cells. RNA samples were dissolved in sterile distilled water and quantified by UV-spectrophotometer.

D. Primers and RT-PCR

PCR Primers for cytokines and β2-microglobulin were from a previous study by Reddy et al. (1996), and primers for G3PDH (Lane 6) were synthesized as described by Yelich et al. (1997) (Table 6.1).

Reverse transcription (RT) was carried out with Expand™ reverse transcriptase (Boehringer Mannheim) according to the manufacturer's instructions. Briefly, cDNA was synthesized at 42°C for 1 hr from 1 μg of total RNA with 50 U of Expand™ reverse transcriptase in a total reaction volume of 20 μl, containing 5 μM oligo$(dT)_{15}$, 1 mM each of dATP, dCTP, dGTP and dTTP, 50 mM Tris-HCl pH 8.3, 40 mM KCl, 5 mM $MgCl_2$, and 0.5% (v/v) Tween 20. Aliquots from the same RT reaction were used to amplify each of the target genes (Table 6.1) for each stage of blastocyst development. A master reaction mixture was prepared for PCR amplification of all target genes in that development stage. Therefore, PCR amplification of all target genes for each stage was

carried out under the same conditions, except that 0.4 µl of cDNA template were used for β2-microglobulin and G3PDH while 10 µl of cDNA template were used for all cytokines. PCR was performed with an initial denaturation step at 94 °C for 2 min, followed by 38 cycles (denaturation at 94 °C for 30 seconds, annealing at 57 °C for 30 seconds and extension at 72 °C for 1 min), and a final extension step at 72 °C for 7 min. All PCR reactions were carried out in a Perkin Elmer GeneAmp 9600 Thermocycler in a reaction volume of 50 µl, containing 1.25U *Taq* polymerase (Gibco/BRL), 0.5 µM of each specific primer, 20 mM Tris-HCl pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$ and 0.2 mM dNTP mixture. RT-PCR amplification was repeated on total RNA from each sample at least twice. Direct PCR amplification of total RNA without prior reverse transcription did not yield products with the predicted sizes for any of the target genes.

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Table 6.1 Primers for cytokines and predicted sizes of PCR products

Target genes	Primer sequences	Sizes of PCR products (bp)	cDNA amount applied for PCR
IL-1	CAGCTATGAGCCACTTCCTG(f) GTCACAGGAAGTTGCGAATC(r)	434	10 µl
IL-2	CAGTAACCTCAACTCCTGCCAC CATCCTGGAGAGATCAGCATTC	199	10 µl
IL-4	GTCTCACATCGTCAGTGC TCATGCACAGAACAGGTC	359	10 µl
IL-6	GGAACGCCTGGAAGAAGATG ATCCACTCGTTCTGTGACTG	470	10 µl
IL-8	TGCAGCTTCATGGACCAG GTACAACCTTCTTCTGCACC	323	10 µl
IL-10	GCTCTATTGCCTGATCTTCC GCACTCTTCACCTCCTCCAC	404	10 µl
TNF-α	CACTGAGAGCATGATCCGAG GGCTGATGGTGTGAGTGAGG	463	10 µl
TNF-β	ATGACACCACCTGGACG TGGTACACAGAGCGCAC	502	10 µl
IFN-γ	TGTACCTAATGGTGGACCTC TCTCTGGCCTTGGAACATAG	378	10 µl
β2 micro-globulin	CTGCTCTCACTGRCTGG ATCGAGAGTCACGTGCT	284	0.4 µl
G3PDH	ACCACAGTCCATGCCATCAC TCCACCACCCTGTTGCTGTA	452	0.4 µl